Universidade de Aveiro Departamento de Biologia
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Botryosphaeriaceae and *Diaporthaceae* species associated with blueberry plants in Portugal

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica do Doutor Artur Jorge da Costa Peixoto Alves, investigador principal do Departamento de Biologia da Universidade de Aveiro, e sob co-orientação científica da Doutora Liliana Tavares dos Santos, investigadora em pós-doutoramento do Departamento de Biologia da Universidade de Aveiro.

“A dream doesn’t become reality through magic. It takes sweat, determination and hard work”

Colin Powell

Dedico este trabalho à minha avó. Uma das pessoas mais importantes da minha vida.
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o júri

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palavras-chave

Vaccinium corymbosum, mirtilo, sistemática, *Diaporthe, Botryosphaeria*, *Neofusicoccum,* patogenicidade, agente patogénico

resumo

O mirtilo (*Vaccinium corymbosum*) é uma planta não nativa, cujo cultivo em Portugal tem crescido representando uma parte importante da economia em algumas regiões do país. O crescimento rápido da produção tem sido acompanhado por um aumento de doenças causadas por agentes fitopatogénicos, particularmente fungos. No entanto, os estudos sobre fungos patogénicos de mirtilo em Portugal são muito escassos. Este estudo pretendeu contribuir para colmatar esta falha, focando as espécies de *Diaporthaceae* e *Botryosphaeriaceae* pois são reconhecidas como agentes patogénicos que afetam plantações de mirtilo a nível mundial.

Para tal foi caracterizada uma coleção de 222 isolados fúngicos obtidos de plantas sintomáticas e assintomáticas. Todos os 222 isolados foram inicialmente submetidos a tipagem por BOX-PCR de modo a avaliar a diversidade genética global da coleção. A partir da análise dos perfis BOX-PCR foram selecionados 81 isolados representativos para identificação molecular por sequenciação e análise da região ITS. A identificação inicial baseada nas sequências da região ITS permitiu a identificação de 13 géneros distintos. De entre estes, *Neofusicoccum* e *Botryosphaeria* (*Botryosphaeriacaeae*) e *Diaporthe* (*Diaporthaceae*) foram os mais abundantes.

Os isolados pertencentes aos géneros *Diaporthe*, *Neofusicoccum* e *Botryosphaeria* foram adicionalmente sujeitos a uma análise de sequências multi locus de modo a permitir a sua identificação correta ao nível da espécie. Foram obtidas sequências do gene que codifica para o fator de alongamento da transcrição 1-alfa (*tef1-α*) para os isolados de *Neofusicoccum* e *Botryosphaeria*. As sequências dos genes que codificam para β-tubulina (*tub*), *tef1-α*, histona (*his*) e calmodulina (*cal*) foram utilizadas para uma análise multi locus das espécies de *Diaporthe*.

A análise filogenética das sequências ITS e *tef1-α* combinadas revelou que os isolados de *Botryosphaeria* pertenciam todos à espécie *B. dothidea*, enquanto os isolados de *Neofusicoccum* pertenciam a três espécies diferentes, nomeadamente *N. australe*, *N. eucalyptorum* e *N. parvum*. *Neofusicoccum parvum* foi a mais abundante de todas as espécies de *Botryosphaeriacaeae* identificadas.

As análises filogenéticas permitiram alocar os isolados de *Diaporthe* a diferentes clados, representando 3 espécies conhecidas (*D. eres*, *D. foeniculina*, e *D. rudis*) e três potenciais novas espécies (*Diaporthe* sp.1, *Diaporthe* sp.2, e *Diaporthe* sp.3). De todas estas, *D. eres* e *Diaporthe* sp. 2 foram as mais abundantes. As três potenciais novas espécies foram caracterizadas em termos morfológicos, capacidade de crescimento a diferentes temperaturas e tipos de *mating*. São apresentadas descrições taxonómicas completas para cada uma delas.

Em testes de patogenicidade usando isolados representativos de cada espécie, todas as espécies de *Diaporthe*, *Neofusicoccum* e *Botryosphaeria* mostraram ser patogénicas para plantas de mirtilo da cultivar *Bluecrop*. *Neofusicoccum parvum* foi a mais agressiva das espécies de *Botryosphaeriacaeae*, enquanto *Diaporthe* sp. 3 foi a mais agressiva das espécies de *Diaporthe* testadas. *Neofusicoccum parvum* foi a única espécie que causou mortalidade de plantas sendo por isso considerada possivelmente como o agente patogénico mais relevante em mirtilo.

Este estudo representa a primeira confirmação da ocorrência, em Portugal, de *D. eres*, *D. foeniculina*, *D. rudis*, *N. australe*, *N. parvum* e *B. dothidea* em mirtilo. Adicionalmente, *N. eucalyptorum*, um agente patogénico comum de *Eucalyptus* spp., é reportado pela primeira vez como agente patogénico de mirtilo a nível mundial. Tal facto representa uma mudança para um novo hospedeiro cujas potenciais implicações futuras em plantações de mirtilo não são conhecidas.

Os resultados apresentados mostram que as espécies de *Diaporthe* e *Botryosphaeriacaeae* são comuns em mirtilo em Portugal. A sua diversidade ao longo do país bem como a sua patogenicidade em relação a outras cultivares de mirtilo merecem ser exploradas no futuro.

keywords

Vaccinium corymbosum, blueberry; systematic; *Diaporthe*; *Botryosphaeria*; *Neofusicoccum;* pathogenicity, pathogenic agent

abstract

The blueberry (*Vaccinium corymbosum*) is a non-native and increasingly cultivated plant in Portugal and represents an important part of the economy of some regions. The rapid growing of blueberry production has been accompanied by an increase of diseases caused by plant pathogens, mainly fungi. However, there is an overall lack of studies concerning blueberry fungal pathogens occurring in Portugal. This study aimed to contribute to fill this gap by focusing on *Diaporthaceae* and *Botryosphaeriaceae* species, which are known pathogens affecting cultivated areas of blueberry worldwide.

For this, a collection of 222 fungal isolates obtained from blueberry plants, including asymptomatic and symptomatic, was characterised. All 222 isolates were initially subjected to BOX-PCR fingerprinting to evaluate their overall genetic diversity. From the cluster analysis performed on BOX-PCR fingerprints 81 isolates representative of each cluster were selected for molecular identification, namely by ITS sequencing and analysis. A primary identification based on ITS sequences allowed identifying 13 different genera with *Neofusicoccum* and *Botryosphaeria* (*Botryosphaeriacaeae*) and *Diaporthe* (*Diaporthaceae*) being the most common.

The isolates identified by ITS sequences as members of *Diaporthe*, *Neofusicoccum* and *Botryosphaeria* were further subjected to multi locus sequence analyses in order to correctly identify them to the species level. Thus, sequences of the translation elongation factor 1-alpha (*tef1-α*) were obtained for *Neofusicoccum* and *Botryosphaeria* isolates. β-tubulin (*tub*), *tef1-α*, histone (*his*), and calmodulin (*cal*) were also used for multi locus sequence analysis of *Diaporthe* species.

Combined ITS and *tef1-α* phylogenetic analyses showed that all *Botryosphaeria* isolates belonged to the species *B. dothidea*, while *Neofusicoccum* isolates belonged to three different species, namely *N. australe*, *N. eucalyptorum* and *N. parvum*. *Neofusicoccum parvum* was the most abundant of all *Botryosphaeriacaeae* species found.

Phylogenetic analyses placed the *Diaporthe* isolates into distinct clades representing three known species (*D. eres*, *D. foeniculina*, and *D. rudis*) and three putative novel species (*Diaporthe* sp.1, *Diaporthe* sp.2, and *Diaporthe* sp.3), with *D. eres* and *Diaporthe* sp. 2 being the most abundant. The putative novel species were fully characterised in terms of morphology, ability to grow at different temperatures and their mating types. Full taxonomic descriptions are given for all of them.

Pathogenicity tests using isolates representative of each species showed that all *Diaporthe*, *Neofusicoccum* and *Botryosphaeria* were pathogenic to blueberry plants of the cultivar *Bluecrop*. Among the *Botryosphaeriacaeae* species*, N. parvum* was the most aggressive while *Diaporthe* sp. 3 was the most aggressive of all *Diaporthe* species tested.

Neofusicoccum parvum was the only species the induced plant death and it is thus regarded as probably the most relevant pathogen of blueberry.
This study represents the first confirmation of the occurrence in Portugal of *D. eres, D. foeniculina, D. rudis, N. australe, N. parvum and B. dothidea* on blueberries. Additionally, *N. eucalyptorum*, a common *Eucalyptus* spp. pathogen, is reported worldwide for the first time as a pathogen of blueberry. This represents a relevant host jump whose potential future implications on blueberry plantations are not understood.
The results presented show that species of *Diaporthe* and *Botryosphaeriacaeae* are common on blueberry plantations in Portugal. Their diversity throughout the country and pathogenicity towards other blueberry cultivars deserve further studies.

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LIST OF ABBREVIATIONS

tef1-α – translation elongation factor 1-α gene

cal – calmodulin gene

his – histone gene

ITS – internal transcribed spacer region

tub – tubulin gene

MAT – mating-type genes

BOX-PCR – BOX sequences – Polymerase chain reaction

UPGMA – Unweighted Pair Group Method with Arithmetic Mean

ML – Maximum likelihood

°C – Celsius degrees

µL – microliter

mL – milliliter

µg – microgram

mg – miligram

µm – micrometre

M – Molar

mM – milimolar

TES – Tris-EDTA-SDS buffer

TE – Tris-EDTA buffer

TAE – Tris-acetate-EDTA buffer

EDTA – Ethylenediaminetetraacetic acid

SDS – Sodium dodecyl sulfate

CIA – Chloroform:Isoamyl alcohol

NH_4OAc – Ammonium acetate

CTAB – Cetyl trimethylammonium bromide

EtBr – Etidium bromide

NaCl – Sodium chloride

dNTPs – deoxy-nucleotide-tri phosphate

rpm – revolutions per minute

$MgCL_2$ – Magnesium chloride

U – enzyme unit

PDA – potato dextrose agar medium

WA – water agar medium

bp – base pair

BLAST – Basic Local Alignment Search Tool

SD – Standard deviation

1. INTRODUCTION

1.1. The genus *Vaccinium*

The blueberry plant is a perennial shrub and belongs to the family *Ericaceae* and genus *Vaccinium* (Hancock & Retamales, 2012). It is a widely cultivated plant across the globe, and in the past few years its popularity has been increasing in several countries as a segment of agricultural production (Elfar et al., 2013; Espinoza et al., 2009; Pizzolato et al., 2014). The fruit is a small berry, with succulent pulp and bittersweet flavour. Its consumption has increased over the last several decades, due to globalisation of the industry and their health benefits (Hancock & Retamales, 2012; Serrado, 2008).

The genus *Vaccinium* includes approximately 450 species from which 40 % are in Asia and Pacific, 26 % in sub-North American continent and 6 % in Europe. In Central and South America, it was found 47 species, 5 in Africa, 6 in Europe, 19 in Japan and 70 in China. More than 250 species, are distributed in Malaysia and Indochina (Fonseca & Oliveira, 2007; Lombard et al., 2014; Michalska & Lysiak, 2015).

Four *Vaccinium* species are regarded as economically important and indigenous to Europe, namely *V. myrtillus* (bilberry), *V. oxycoccus* (cranberry), *V. uliginosum* (bog bilberry) and *V. vitis-idaeae* (lingonberry). *Vaccinium macrocarpon* (American cranberry) and *V. corymbosum* (highbush blueberry), which were introduced from North America, are also commercially cultivated in Europe (Lombard et al., 2014). Even though there are such a wide variety of blueberry plants, within them we can define three larger groups according to their commercial value – highbush, rabbiteye and lowbush (Spiers, 1998).

Portugal counts with 5 wild blueberry species. *Vaccinium myrtillus*, *V. vitis-idea* and *V. uliginosum* are found in the north region of the country; including Serra do Gerês, Trás-os-Montes and Serra da Estrela (Fonseca & Oliveira, 2007). Regarding the Portuguese islands there are 2 more native species, *V. padifolium* from Madeira and *V. cilindraceum* from Azores (Fonseca & Oliveira, 2007).

1.2. Blueberry benefits

Since pre-history, there are evidences of consumption of blueberries, fresh or transformed. The oldest remnants are dehydrated traces of a kind of drink or jam found in pottery in Denmark dated from Bronze Age (Fonseca & Oliveira, 2007). The essential elements that gave blueberry popularity in Europe (potassium, calcium, phosphorus, magnesium, aluminium, boron, copper, iron, sodium, manganese and zinc) are important components of blueberries, while low content of sodium turns them especially suitable for human consumption (Vollmannova et al., 2014). The fruit is also known to be a food resource with bioactive compounds such as anthocyanins, vitamins, flavonoids, polyphenols, ascorbic acid and other bioactive organic substances holding antioxidant and anti-inflammatory activities (Cuce et al., 2013; Kim et al., 2015; Ruzi et al., 2012; Song, 2015). Additionally, this fruit has the potential to inhibit the proliferation and severity of certain cancers and vascular diseases (Caspersen et al., 2016; Kim et al., 2015).

Some health benefits hold by blueberry consumption include (Mirtilusa, 2017):

a. Decreased formation of blood clots;
b. Reduced digestive tract inflamation;
c. Reinforces memory;
d. Improves night vision and tired sight;
e. Prevents cardiovascular disease;
f. Prevents several types of cancer;
g. Slows the brain aging in Alzheimer's patients;
h. Indicated in diets for hypertension;
i. Protects the skin;
j. Accelerate healing;
k. Anti-inflammatory properties.

1.3. Blueberry production worldwide

Most countries producing blueberry (*Vaccinium corymbosum*) are located in the northern hemisphere, and the USA is the main producer and consumer at world level (Hung et al., 2016; Larach et al., 2009; Michalska & Lysiak, 2015; Song, 2015; Strik, 2016). Blueberry is a commercially important woody plant which has been cultivated in the Netherlands since 1920s, and nowadays widely spread to many other countries such as Canada, France, Germany, Italy, Korea, Spain, Portugal and USA, due to its fruit known to contain high amounts of antioxidants beneficial for human health (Hancock & Retamales, 2012; Hung et al., 2016; Prodorutti et al., 2007).

In North America, there is a great abundance of blueberry species and the fact that they are very common and used by the native ones, made that the habit of its consumption passed quickly to the European colonisers (Madeira, 2016). For this reason, the first commercial plantations took place in the USA, progressing endlessly after 1940, following an insatiable increase in consumption and rapid progress regarding the genetic improvement (Hancock & Retamales, 2012).

In Australia and New Zealand, the first blueberries were planted the 1960s and 1970s, primarily as a crop for export markets (Hancock & Retamales, 2012). In Asia, the first blueberries were planted in the 1950s. A significant industry emerged in Japan in late 1980s (180 ha), but only a few blueberries were planted in China until recently (Hancock & Retamales, 2012).

It was only in 1923 (as soon as the breeding program in the USA started) that the first blueberry plants were imported into the Netherlands. In the 1950's, Germany had already 50 hectares planted. In the 80's, the culture began professionally in the Iberian Peninsula (Madeira, 2016). Blueberry hectarage remained small across Europe until 1990, with the most planting being done in Italy, France, Germany and the Netherlands (Hancock & Retamales, 2012; Madeira, 2016). The USA, South America and Canada are responsible for about 88.4 % of the worldwide blueberry production, while Europe represents 10.9 % of the global production and Oceania represents only 0.6 % (FAOSTAT, 2017).

Data from 2014 show that the USA is still the first blueberry producer at world level with an annual production of 262 539 ton, followed by Canada and

Mexico with 182 275 and 18 031 tons respectively (FAOSTAT, 2017). In 2014, in Europe, Poland and Germany had similar productions of approximately 12 000 tons. Portugal appears in the end of the raking with a production of 267 tons (FAOSTAT, 2017).

1.4. Blueberry production in Portugal

The true incentive given to the blueberry crop in Portugal occurred in the 80's when the Lockhorn Foundation, at the invitation of the *Fundação Barbosa de Quadros*, carried out a prospective study in the central region of Sever do Vouga and Trancoso, seeking to confirm the possibility of the early production to supply the markets of the Nordic countries (Madeira, 2016; Madureira et al., 2014). The municipality of Sever do Vouga led the area planted until 2012, at which time the crop expanded at an extremely fast step for several regions of the country, without removing to that region the title of Capital of the Blueberry (Brazelton, 2011; Madeira, 2016).

The last years have witnessed an increase of the cultivated area, which evolved from 75 hectares in 2011 to 1481 hectares in 2016 (INE, 2012, 2017), which in 5 years represents an increase of 1975 % (Table 1). According to the Portuguese Agricultural Statistics of 2014, the blueberry production increased from 1824 tons in 2014 to 4436 tons in 2015 (INE, 2014). Despite the higher production and cultivated area in 2016 than the previous years, the year 2011 had the highest production per hectare, with a productivity of 9.3 tons / ha (INE, 2016).

Table 1 – Yield and cultivated area of blueberry in Portugal between 2011 and 2016 (Adapted from INE, 2012 - 2017).

Year	Yield (ton)	Area (ha)	Productivity ton/ha
2011	700	75	9.3
2012	1437	211	6.8
2013	1429	534	2.7
2014	1824	823	2.2
2015	4436	1325	3.3
2016	6572	1481	4.4

The production of blueberries in Portugal, between 2011 and 2016, has increased year on year (Table 1). In 2011, Portugal had an annual production of 700 tons of blueberry and the highest productivity in this time frame. In 2012, this production has duplicated to 1437 tons. The rise in production over the years is largely due to the adopted agricultural policies and financial support with the purpose of encouraging the implementation of new farmers to produce fresh berries. However, this fact may explain the increase of the cultivated area, but not the productivity since the crop needs in average 3 years to begin the production.

In Portugal, the progressive growth and development of the sector food and the influence of other European countries, has introduced new eating habits, such as consumption of berry fruits, in which is included the blueberry. On the other hand, the country, due to its soil and climate conditions, has a high potential for blueberry crop. Sever do Vouga (Central-North Portugal) and Zambujeira do Mar in Alentejo (Southern Portugal) are the main blueberry producing regions (Madureira et al., 2014). Driscoll's, a company from California which is the world's largest producer of strawberries, raspberries, blackberries and blueberries has recently found in Alentejo the ideal climate conditions to produce such fruits. In the last ten years, it has invested around 17 million euros in the country. In 2015, they announced an estimated investment of 1.5 to 3 million euros which would allow to double the area of blueberry cultivation, ranking Portugal in the largest producer for the markets of Europe, Middle East and Africa (Nunes et al., 2017).

In the last edition of the Fruit Logistic 2017 that took place in Berlin, (a specialised annual trade show and conference event for fruit and vegetable business) the Portuguese Minister of Agriculture, Luis Capoulas Santos advanced that in 2015 the exports of fresh berries, including blueberry, dethroned pear (cultivar '*Rocha*') with 90.6 million euros and 86.5 million euros respectively. The minister has also highlighted the importance of fresh fruits that have been an increasingly impact in the Portuguese agriculture and economy (Nunes et al., 2017).

Although blueberry and other berries have been increasingly produced in Portugal, there are other crops that represent a bigger weight in the economy of the country. Among the most produced commodities are vegetables such as

potato, and tomato for industry, following cereals like maize and rice (FAOSTAT, 2017; INE, 2017). From the fruit trees, it can be highlighted the orange, apple, pear, kiwi and chestnuts. Olive production for olive oil and vineyard represent equally an economic importance to the country (INE, 2017). At last, it appears the fresh berries such as blackberry, blueberry, gooseberry and raspberry (Oliveira & Fonseca, 2007a). Among these fruits, and between 2013 and 2016, blueberry was the one that represented the highest cultivated area. However, it is the raspberry that has the highest production over the years (INE, 2017). Such fact may be explained because the production of raspberry occurs in conditions of controlled environment all year (Oliveira & Fonseca, 2007a). Nevertheless, the highest cultivated area of blueberries may explain the greater interest in the consumption and exportation of this berry fruit over others.

1.5. Blueberry cultivars

Within such a wide variety of blueberry plants, we can define three larger groups according to their commercial value – highbush, rabbiteye and lowbush (Hancock & Retamales, 2012; Spiers, 1998). These groups are distinguished by their polyploidy levels. Highbush is a group of tetraploid plants. The main species of this group is *Vaccinium corymbosum*, but *Vaccinium australe* and *Vaccinium darrowi* can also be included (Michalska & Lysiak, 2015; Ortiz et al., 1992). Plants belonging to rabbiteye group are hexaploid. The main species of this group is *Vaccinium ashei* (Ortiz et al., 1992; Spiers, 1998). Lowbush plants are diploid and produce the smallest fruits, which go mainly to the transformation industry for jams and other products. *Vaccinium angustifolium* is the most known species of this group (Ortiz et al., 1992).

The highbush blueberries, which include northern and southern cultivars are the most cultivated worldwide (Hancock & Retamales, 2012; Michalska & Lysiak, 2015). Within highbush blueberries, the northern highbush is the variety more cultivated and includes the cultivars: *Duke*, *Bluecrop, Bluegold, Huron, Chandler, Draper, Legacy, Liberty, New Hanover, Cipria, Elliot, Aurora, Goldtraube, Spartan, Brigitta* and *Ozarkblue* (Martins et al., 2015). The southern highbush variety

includes: *O'neal*, *Star*, *Misty, Biloxi*, *Sharpblue, Suziblue, Rebel and Camelia* cultivars (Martins et al., 2015).

The rabbiteye variety includes the *Skyblue*, *Powderblue, Ochlockonee* and *Columbus* cultivars and compared to the previous group, it produces smaller fruit with lower quality (Hancock & Retamales, 2012; Martins et al., 2015). The lowbush variety is considered to have the half-high cultivars including *Chippewa*, *Northblue*, *Northcountry*, *Northsky*, *Polaris*, *St. Cloud* and *Superior* (Hancock & Retamales, 2012). In Portugal, the cultivars *Duke, Bluecrop, Ozarkblue* and *Goldtraub* are among the most cultivated (Mirtilusa, 2017).

1.6. Blueberry diseases

Currently, blueberry has an increased value all over the world, with thousand hectares spread across diversified soils and climates (Elfar et al., 2013). This extensive range of soil and climates may enhance the development of new diseases that do not occur in the natural habitat of blueberry plants, mostly due to anthropological activities (Stukenbrock, 2016). For example, in Chile disease incidence was between 15 % and 45 % in the past few years (Espinoza et al., 2008, 2009). Also, the rapid growth of blueberry production has been associated with the emergence of several diseases caused by plant pathogens that limit cultivation (Lombard et al., 2014). These pathogenic agents can be bacteria, viruses or fungi, and all of them are described worldwide in blueberry plants (Espinoza et al., 2008, 2009; Kałużna et al., 2013; Szmagara, 2009). However, the agents causing diseases can also be insects or nematodes (Hancock & Retamales, 2012; Polavarapu et al., 2007; Shutak & Gough, 1982). When disease is present in blueberry plantations, the impact is devastating – spreading from plant to plant, these agents can severely reduce harvest of blueberries and lead to significant losses (Brewer et al., 2014; Elfar et al., 2013; Lombard et al., 2014)

1.6.1. Bacterial diseases

Vaccinium corymbosum is infrequently infected by bacterial pathogens (Kałużna et al., 2013). However, Canfield et al. (1995) described that

Agrobacterium tumefaciens can cause pea-sized galls on low branches and at the base of canes (as cited in Pscheidt & Ocamb, 2016). Since blueberries are grown on acidic soils, *A. tumefaciens* infections are uncommon (once the crown gall bacterium does not grow in acidic environments) (MSU, 2016; Prodorutti et al., 2007). The only bacterial pathogen described in Poland was the tumorigenic *Agrobacterium* spp. causing crown gall (Kałużna et al., 2013).

In the USA and other countries, bacterial canker caused by *Pseudomonas syringae* was observed. *Pseudomonas andropogonis* was also reported in New Jersey causing leaf spot and affecting blueberry growth (Kałużna et al., 2013).

Xylella fastidiosa, a newly identified disease has the potential to cause major damage to highbush blueberries in the southeastern of USA (Kałużna et al*.,* 2013; MSU, 2016). This species has been reported in Spain, France and Italy causing damages in olives. Its presence in Portugal has not been detected yet (SNAA, 2017).

1.6.2. Viral diseases

At least 6 viruses have been found in highbush blueberry plantations in the Pacific Northwest: *Blueberry mosaic virus*, *Blueberry red ringspot virus*, *Blueberry scorch virus*, *Blueberry shock virus*, *Tobacco ringspot virus*, and *Tomato ringspot virus* (Martin et al*.,* 2012). The cultivars *Berkeley, Bluegold, Bluetta, Duke, Liberty, Aurora, Pemberton, Reka*, and *Elliott* are particularly susceptible and the virus spreads rapidly (Pscheidt & Ocamb, 2016). Transmission of the *Blueberry scorch virus* (BlScV) occurs when pollinators, especially honeybees, transfer infected pollen to flowers on healthy plants (Pscheidt & Ocamb, 2016).

1.6.3. Nematodes

Stubby root nematodes (*Paratrichodorus* species) and root lesion nematodes (*Pratylenchus* species) have been found in root zone soil of blueberry plants in North America. However, the reproductive potential and damage caused by these nematodes to blueberries is unknown (Forge et al., 2012).

In Portugal, there are no published reports or information regarding bacterial and viral diseases on blueberries as well as the occurrence of nematodes (Madeira, 2016).

1.6.4. Insects

Regarding insect's (arthropods) attack on blueberry crop in Portugal, it can be highlighted the presence of aphids (*Myzus persicae*, *Aphis gossypii* and *Aphis fabae*), thrips (*Thrips flavus*, *Trhirps tabaci*), scale insects (*Icerya pruchasi* and *Quadraspidiotus perniciosus*), black vine weevil (*Otiorhynchus sulcatus*), tussock moth caterpillar (*Orgya antiqua*), beetles (*Melolonta* spp.) and flies (*Dasineura oxycoccana*, *Prodiplosis vacinii* and *Drosophila suzukii*) (Hancock & Retamales, 2012; Madeira, 2016). Most of these insects were introduced into Europe and consequently in Portugal, through imported plants from diseased nurseries (Madeira, 2016). Still, mites (arachnids) are also able to cause damages on blueberry crop in Portugal (Madeira, 2016).

Drosphila suzukii, the spotted wing drosophila is a small fly that has recently been reported in Portugal (Hancock & Retamales, 2012; Moreira, 2015). It has a wide host range and can attack many fruit crops, including small fruit crops and fruit trees such as *Actinidia* spp., *Diospyros kaki*, *Malus domestica*, *Prunus avium*, *Prunus persica*, *Rubus armeniacus*, *Vaccinium* spp. and *Vitis vinifera* (EPPO, 2010; Moreira, 2015).

In Portugal, *D. suzukii* was reported in 2012 and it is important for blueberry production once the infested fruit begin to collapse around the feeding site, which also increases fungal or bacterial infections leading therefore to production losses (EPPO, 2010; Madeira, 2016; Moreira, 2015).

1.6.5. Fungal diseases

Among the microorganisms causing diseases on blueberries all over the world, fungi play a significant role (Money, 2016; Szmagara, 2009). Losses from blueberry fungal diseases depend on season climatic conditions and on the susceptibility of the cultivar (Barrau et al., 2002).

Several fungi are associated with stem canker, dieback, blight and other diseases in blueberry which are a primary factor limiting both longevity and production (Espinoza et al., 2009). Some of these fungi include species of *Alternaria*, *Armillaria, Botryosphaeria*, *Botrytis*, *Diaporthe*, *Exobasidium*, *Godronia*, *Lasiodiplodia*, *Neofusicoccum, Fusarium, Pestalotiopsis, Sclerotinia*, *Truncatella, Valdensinia,* and others (Brewer et al., 2014; Diogo et al., 2016; Elfar et al., 2013; Espinoza et al., 2008, 2009; Farr et al., 2002a; Lombard et al., 2014; Perez et al., 2014; Produrotti et al., 2009; Umemoto et al., 2007; Xu et al., 2015; Wright et al., 2012; Wright et al., 2014; Wright & Harmon, 2010;).

The rust fungus *Naohidemyces vaccinii*, also referred as *Pucciniastrum vaccinii* or *Thekospora vaccinii* has been added to A2 list of quarantine diseases of EPPO and it has been reported in Portugal, Australia, Europe, Argentina, Asia, Mexico, Canada and the USA (Chicau, 2015; EPPO, 2017b, 2017c). Reddish brown spots appear on leaves, which turn yellow and drop prematurely (MSU, 2016).

Armillaria species induce root disease on wide-ranging plants and cause economic losses. Since *Armillaria* spp. is not a pathogen specific of *V. corymbosum*, the presence of infected roots (of various plant species) is an important and expected source of inoculum (Chicau, 2015; Produrotti et al., 2007). *Armillaria mellea, A. ostoyae* and *A. gallica* have been reported on highbush blueberry in the USA (Prodorutti et al., 2006). *Armillaria* root rot is rare on blueberries in the USA, but can cause serious damage where it occurs (MSU, 2016). In Italy, the fungus has been found since 2003 (Prodorutti et al., 2009).

In Argentina, the species *Fusarium solani*, *F. proliferatum* and *F. acuminatum* were identified in blueberries as causing agents of branch blight, premature branch death, discoloration of the leaves, spots along the stem and root and stem rot (Perez et al., 2007, 2011; Wright et al., 2014). *Fusarium oxysporum* and *F. acuminatum* have also been reported in China as causing wilt of leaves and fruit rot respectively (Farr & Rossman, 2017). These fungi can remain in the soil under the form of mycelium or spores, even in the absence of the host (Chicau, 2015).

Anthracnose fruit rot (causal agent, *Colletotrichum acutatum*; sexual morph: *Glomerella acutata*) is an important disease problem in USA and can appear on fruit before harvest (ripe rot) but more often appears as a postharvest fruit rot (Polashock et al., 2005; Pscheidt & Ocamb, 2016). *Colletotrichum gloeosporioides* was first reported in China in 2008 as causing stem and leaf spots on blueberries (Xu et al., 2013). Symptoms like water soaked lesions and rotten fruits associated with anthracnose were also reported in Korea during 2008 as occurring in blueberries (Kwon et al., 2008).

In 2002, *Sclerotinia sclerotiorum* was reported in Japan as causing rotted flowers and blighted tips and leaves on highbush blueberry (Umemoto et al., 2007). *Sclerotinia sclerotiorum* was first reported in Argentina in 2007 and in 2012 it was described as causing a postharvest fruit rot of blueberry in Europe (Lopez et al., 2015; Perez et al., 2011b).

Mummy berry disease is caused by the fungus *Monilinia vaccinia-corymbosi* and it occurs in commercial and native *Vaccinium* spp. throughout North America (Lehman et al., 2007). In Austria, Gosch (2006) had also reported this fungus on *V. corymbosum* (as cited by Pscheidt & Ocamb, 2016). The disease is characterised by blighting vegetative and flower buds and by causing rot of the fruit and shoots (Burchhardt & Cubeta, 2015; Lehman et al., 2007; MSU, 2016)

Botrytis blossom blight caused by the fungus *Botrytis cinerea* can be the source of severe crop losses of rabbiteye blueberry and it is usually unimportant on highbush blueberry (Smith, 1998). In this last variety, the fungus is capable of living as a saprophyte. *Botrytis cinerea* infects flowers, fruits and young shoots of blueberries (Barrau et al., 2002).

In July 2013, a new disease occurred on leaves of highbush blueberry in Japan. The causal fungus was identified as *Valdensia heterodoxa*. Leaves presented brown spots that developed into rotting and leading to a premature defoliation (Nekoduka et al., 2012).

Exobasidium maculosum, the causal agent of leaf and fruit spot of blueberry, is considered an emerging disease that has been reducing fruit quality in some plantations since 2011 (Brewer et al., 2014; Stewart et al., 2015). It occurs sporadically in North Carolina and Canada. Small green spots on leaves

and fruit appear after bloom and near harvest, a dense white layer of spores develops on the undersides of leaf spots (MSU, 2016).

Symptoms of blueberry stem diseases are usually complicated in field surveys, and it was found that *Pestalotiopsis* spp. and *Diaporthe* spp. sometimes coexist in the same infected twigs (Elfar et al., 2013; Espinoza et al., 2008, 2009; Xu et al., 2016). According to Nekoduka et al. (2012), *Pestalotiopsis* spp. may also cause leaf spot. In Chile, Espinoza et al. (2008) identified the species *Pestalotiopsis clavispora*, *P. neglecta* and *Truncatella angustata* associated with blueberry exhibiting cankers. Also in China, *P. clavispora* was first reported as causing twig blight on *Vaccinium corymbosum* (Chen et al., 2016).

Root rot is present in the main production areas worldwide, and several species of the oomycete *Phytophthora* (*Phytophthora cinnamomi, P. citrophthora, P. nicotinae* and *P. palmivora*) have been identified (Larach et al., 2009; Tamietti, 2003). Soils with poor drainage are favourable to the development of this oomycete (Chicau, 2015). In Italy, *P. cinnamomi* was described in 1996 as causing inhibition, pale green leaves, premature defoliation, and root and crown rot in *V. corymbosum* (Tamietti, 2003). Larach et al. (2009) reported for the first-time *Phytophthora* root rot in Chile occurring in highbush blueberries. *Phytophthora cinnamomi* was first reported in China in 2014 as causing root and stem rot of blueberries (Lan & Yao, 2016). In California, this oomycete was also described as causal agent of chlorotic, necrotic and blackened roots in 2015 (Shands et al., 2016).

Alternaria fruit rot occurs in most blueberry-growing regions and it is caused by several *Alternaria* species. As fruit ripens, the first symptom is a shrinking berry (Mehra et al., 2013; MSU, 2016; Zhu & Xiao, 2015). Sometimes when leaf lesions occur, are circular to irregularly and surrounded by a rose-coloured to brown border (Pscheidt & Ocamb, 2016). In China in 2006, it was reported *Alternaria tenuissima* on blueberries, causing reddish, circular spots on leaves and stems which led to the development of small and insignificant cankers (Luan et al., 2007). In Argentina, this fungus was first documented in 2004 as causing leaf twig blight and fruit rot of highbush blueberry and in Western Australia was reported in 2013 (Wright et al., 2004; You et al., 2014). Both *A. tenuissima* and *A. alternata* have

been responsible for postharvest fruit rots of blueberries (Mehra et al., 2013; Zhu & Xiao, 2015). In 2012, it was first documented the appearance of leaf spots on blueberries, caused by *Alternaria* species in South Korea (Kwon et al., 2014). The genus *Alternaria* is currently divided into 26 sections. Species within sect. *Alternaria* have been mostly described based on morphology. Once *Alternaria* species are morphologically indistinguishable, some molecular methods have been developed to distinguish all species within the sect. *Alternaria*. However, these molecular methods have been insufficient, since molecular variation between species is roughly insignificant (Woudenberg et al., 2015; Zhu & Xiao, 2015). Zhu & Xiao (2015) have also evidenced that *Alternaria alternata* and *Alternaria tenuissima*, both potentially pathogenic to blueberries, were clustered in the same clade after phylogenetic analysis, which turns difficult to distinguish both species. The fact that *Alternaria tenuissima* is being incorrectly listed as *A. alternata* may explain this difficulty (You et al., 2014).

Diaporthe vaccinii (asexual morph*: Phomosis vaccinii*) and other *Diaporthe* species have been described as causing dieback and fruit rot in *V. corymbosum* (blueberry) and *V. macrocarpon* (cranberry) plants in North America (Elfar et al., 2013; Lombard et al., 2014). In USA, field mortality can go up to 50% in more than 2 ha of property when progressive twig dieback occurs (Farr et al., 2002a).

Diaporthe baccae and *D. sterillis* were first documented as blueberry pathogens in Europe, causing cankers, brown lesions on stems and twigs and consequently twig blight. *Diaporthe eres* and *D. rudis* were also first reported on blueberries from symptomatic material surveyed from the Netherlands (Lombard et al., 2014). *Diaporthe ambigua*, *D. australafricana* and *D. foeniculina* were first reported in blueberries in Chile, as causing reddish-brown necrotic lesions (Elfar et al*.,* 2013).

Species of *Botryosphaeriaceae* are common pathogens of blueberries (Espinoza et al., 2008; Tennakoon et al., 2017b). Blueberry stem blight, caused by *Botryosphaeria*, *Neofusicoccum* and *Lasiodiplodia* species, is the most destructive disease affecting blueberry production worldwide (Chang-Nan, 2016; Wright & Harmon, 2010; Xu et al., 2015). Although studies carried out by INIAV (Instituto Nacional de Investigação Agrária e Veterinária) have shown that *Diaporthe* and

Botryosphaeriaceae species are the second (20.7 %) and third (17.7 %) more frequent species isolated from blueberry samples in Portugal between 2013 and 2016, little attention is given to infections caused by these species in the country (Diogo et al., 2016). They cause cankers and stem blight, but it is the root rot caused by *Phytophthora* species that displays the biggest risk for blueberries (Diogo et al., 2016; Madeira, 2016).

Phytophthora, *Fusarium* and *Armillaria* species which cause root rot, are the more prominent and recognised diseases in Portuguese blueberry plantations (Madeira, 2016). Mirtilusa, a Portuguese society of fruit and vegetable producers has confirmed that *Armillaria* have been considered one of the major diseases occurring in blueberries in Portugal. Such fact is due to some blueberry plantations are placed in soils that were previously belonging to vineyards or forestry containing host species of this fungus such as *Quercus*, *Pinus*, *Castanea* and *Eucalyptus* (Bragança et al., 2004; Diogo et al., 2016; ICNF, 2015; Mirtilusa, 2017; Neno, 2004). Organic mulch may also promote the growth of root pathogens since *Armillaria* has been found on coniferous bark used as mulch in highbush blueberry plantings and so it may enhance the infection by this pathogen (Hancock & Retamales, 2012; Produrotti et al., 2006).

Additionally, Agriminho, a Portuguese organisation that provides services to small fruits plantations, has been warning to the enormous risks that *Phytophthora* represents to the blueberry culture in Portugal. This organisation also explains that the first form of infection is the irrigation water coming from wells, ponds or rivers; in other cases, the soil itself presents the oomycete resulting from other plantation, mostly shrubs or trees (Marketing Agricola, 2016).

Also in Portugal, *Fusarium* species on blueberries are associated with root rot, chlorosis of the leaves that turn brown and end up falling, leading to the drought of plant. However, these species may be in association with other fungi, co-habiting in the same plant and whose symptoms can be misunderstood (Diogo et al., 2016).

In European countries, shoot dieback, twig blight, stem cankers, root and fruit rots are among the diseases affecting *Vaccinium* spp. and are caused by different pathogens (Lombard et al., 2014; Vilka & Volkova, 2015).

1.6.5.1. Insights into the genus *Diaporthe*

Diaporthe (including the *Phomopsis* asexual morph) belongs to kingdom Fungi, phylum Ascomycota, class Sordariomycetes, order Diaporthales and family *Diaporthaceae* (Dissanayake et al., 2017; Maharachchikumbura et al., 2016).

The ascomycete genus *Diaporthe* includes plant pathogens and endophytes that are most commonly seen as their asexual morph (*Phomopsis*), infecting an extensive variety of hosts (Diogo et al., 2010; Santos & Phillips, 2009; Udayanga et al., 2011).

In the literature, the names seem to be very confusing, but Udayanga et al. (2011) gave preference to use the asexual morph name since this is most common in nature and it is also applied to many important diseases. To Elfar et al. (2013), the older name *Diaporthe* is preferred over its asexual morph *Phomopsis*. Recently, Rossman et al. (2015) recommended the use of the name that has priority, *Diaporthe*, and that is the one currently followed.

Over 1000 species names are described in the genus *Diaporthe* (including the asexual morph), most of them based on host association (Santos et al., 2010). However, biology and life style of some of these species are still mysterious (Vilka & Volkova, 2015).

Diaporthe species are responsible for diseases on a wide range of woody plants and non-woody plants, some of which are economically important worldwide, causing root and fruit rots, dieback, stem cankers, leaf spots, blights, decay and wilting (Dissanayake & Phillips, 2017; Elfar et al., 2013; Farr et al., 2002; Gomes et al*.,* 2013). Species of *Diaporthe* occur mostly as endophytes that under some circumstances behave as pathogens (Farr et al., 2002a; Udayanga et al., 2011). Although *Diaporthe* spp. are widely distributed and mostly known as plant-associated fungi, it has been reported an opportunist infection caused by a *Diaporthe* species in a heart transplant patient (Rakita et al., 2017).

For this genus, morphology has been the basis of taxonomic studies. This genus is characterised by the production of 3 types of conidia, namely alfa (α), beta (β) and gamma (γ) (Gabler et al., 2004; Udayanga et al., 2011). However, since the identification of some *Diaporthe* species is difficult and complicated because many species have similar morphological characteristics, confirmation of

taxonomic identity by molecular techniques is nowadays needed (Farr et al., 2002b; Michalecka et al., 2016; Udayanga et al., 2011; Vilka & Volkova, 2015). Recently, DNA sequence comparisons as well as a multi-locus phylogenetic analysis combining ITS, *tef1-α*, *tub*, *cal* and *his* have become the most effective tool to identify cryptic fungal species in *Diaporthe*, known to be highly complex (Dissanayake et al., 2017; Gomes et al., 2013; Santos et al., 2017; Udayanga et al., 2011)

Diaporthe vaccinii, the causal agent of dieback of blueberry, is native to North America (Lombard et al., 2014). Death of stems and twig blight caused by *D. vaccinii* is a serious economical disease and it is a threat to highbush blueberry (*V. corymbosum*), lowbush blueberry (*V. angustifolium*), and American cranberry (*V. macrocarpon*) in the USA and worldwide (Narouei-Khandan et al., 2017; Szmagara, 2009). In Europe, the European cranberry (*V. oxycoccos*), rabbiteye blueberry (*V. virgatum*), bilberry (*V. myrtillus*), lingonberry or cowberry (*V. vitis-idaea*) and bog bilberry (*V. uliginosum*) are among the potential wild host species of *D. vaccinii* (Narouei-Khandan et al*.,* 2017).

Wilting of leaves on affected shoots, browning of flower buds and discoloration of the xylem characterise the twig blight in blueberries (Narouei-Khandan et al*.,* 2017; Polashock & Kramer, 2006; Weingartner & Klos 1975). Tissue around the canker can appear silvery and spotted with black dots. The pathogen overwinters on infected dead twigs and other plant materials. The disease is difficult to distinguish from symptoms caused by other factors such as herbicide or fungicide injuries, stress caused by heat and drought, frost damages and nutritional deficits (Chicau, 2015; Gabler et al., 2004; Pscheidt & Ocamb, 2016). Also, it can be mistaken with bacterial canker or even with other fungi such as *Pestalotiopsis*, *Colletotrichum* and *Botryosphaeriaceae* species (Michalecka et al., 2016; Pscheidt & Ocamb, 2016).

Since many years there are regular imports to Europe of *Vaccinium* plants for planting from North America. Although conidia can be dispersed to other hosts through rain and irrigation water, natural spreading of spores of *D. vaccinii* only occurs over short distances (Jeger et al., 2017; Narouei-Khandan et al., 2017). The most probable way to introduce *D. vaccinii* into Europe is the import of potted

highbush blueberry plants from countries where *D. vaccinii* may be present (Lombard et al., 2014; Jeger et al., 2017; Michalecka et al., 2016). The climate may also affect the establishment of the pathogen (Jeger et al., 2017; Narouei-Khandan et al., 2017). Because of the risk of further spread, it has been added to A2 list of quarantine diseases of the European and Mediterranean Plant Protection Organization (EPPO), and the corresponding national list of countries including Lithuania and Germany (EPPO, 2017b; Gabler et al., 2004). At present, in Europe there have been confirmed records of *Diaporthe vaccinii* from Lithuania, Poland, Germany, Latvia, and the Netherlands, apparently introduced with American *Vaccinium* cultivars (EPPO, 2017a; Lombard et al., 2014; Michalecka et al., 2016; Vilka & Volkova, 2015). Outside Europe, it has also been found in Russia and China (Narouei-Khandan et al., 2017). However, Jeger et al. (2017) stated that Latvia is the only European country where the pathogen is officially present.

1.6.5.2. Insights into the family *Botryosphaeriaceae*

The *Botryosphaeriaceae* was introduced by Theissen and Sydow in 1918 (Phillips et al., 2013). Over the years, there was some confusion on the taxonomy and systematics of this group (Crous et al., 2006). The application of DNA sequencing data allowed determining the phylogenetic relationships within the group and a better definition of species (Crous et al., 2006; Phillips et al., 2013; Wet et al., 2008).

Botryosphaeriaceae fits in the kingdom Fungi, phylum Ascomycota, Class Dothideomycetes and order Botryosphaeriales (Crous et al., 2016).

This family includes important plant pathogens infecting a wide variety of hosts of angiosperms and gymnosperms including ornamental, forest, mangrove and fruit trees (Alves et al., 2013; Crous et al., 2006; Osorio et al., 2017; Slippers & Wingfield, 2007; Zlatkovic et al., 2016). These fungi are also known to occur worldwide in asymptomatic plant tissues living as endophytes on both cultivated and native plants (Alves et al., 2013; Barradas et al., 2016; Espinoza et al., 2009; Slippers & Wingfield, 2007). Species of *Botryosphaeriaceae* are usually regarded as latent opportunistic pathogens, persisting in healthy tissues and causing diseases on plants that have been exposed to environmental stress, of which

drought is the most frequently reported (Alves et al., 2013; Barradas et al., 2016; Crous et al., 2017; Osorio et al., 2017; Phillips et al., 2013; Slippers et al., 2007; Wright & Harmon, 2010). Additionally, some *Botryosphaeriaceae* species are known to cause opportunistic infections in humans (Úrbez-Torres, 2011; Yang et al., 2017).

The current interest in these fungi is certainly related to their ability to cause severe diseases in plants from natural landscapes as well as in agriculture and forestry (Crous et al., 2017; Slippers et al., 2017; Zlatkocic et al., 2016).

Botryosphaeriaceae is known to include many species phylogenetically related and morphologically similar, which makes the identification using morphological methods difficult (Pavlic et al., 2009; Phillips et al., 2013; Zlatkovic et al., 2016). The application of DNA sequence data and phylogenetic analyses have been crucial for systematic studies of this family (Philips et al., 2013). The current use of the genetic markers, ITS and *tef1-α*, have helped the identification and discrimination of species in the *Botryosphaeriaceae* family (Phillips et al., 2013; Yang et al., 2017; Yu et al., 2013).

Different *Botryosphaeriaceae* species have been recorded as the main causal agents responsible for blueberry stem diseases in different countries such as Chile, Argentina, USA, New Zealand, Turkey, China and Korea (Choi et al., 2012; Dil et al., 2013; Espinoza et al., 2009; Philips et al., 2006; Sammonds et al., 2009; Wright et al., 2012; Wright & Harmon, 2010; Xu et al., 2015; Yu et al., 2012). Stem canker and stem blight of highbush blueberry (*Vaccinium corymbosum*) and rabbiteye (*V. ashey*) is caused by *Botryosphaeria dothidea* (asexual morph: *Fusicoccum aesculi*) and *Botryosphaeria corticis*, considered a threat to blueberry plantations in the USA (Creswell, 1987; Milholland, 1972; Perez et al., 2014 Phillips et al., 2006; Wright & Harmon, 2010). Among *Neofusicoccum* species causing stem blight, dieback, twig blight and discoloration of the vascular tissues on blueberries are *N. arbuti*, *N. ribis*, *N. australe* and *N. parvum*. These species were previously described as pathogenic occurring on blueberries in Chile, China, Korea, Argentina, California, México, New Zealand and Spain (Boyzo-Marin et al., 2016; Castillo et al., 2013; Choi et al., 2012; Espinoza et al., 2009: Koike et al., 2014; Sammonds et al., 2009; Tennakoon et al., 2017; Wright & Harmon, 2010;

Wright et al., 2012; Xu et al., 2015). *Lasiodiplodia theobromae* was also reported in Florida and China as causing stem blight and dieback of blueberries (Espinoza et al., 2009; Wright & Harmon, 2010; Xu et al., 2015).

2. SCOPE OF THE WORK

At present, the blueberry is an increasingly cultivated plant in Portugal and their fruits have high economical relevance. The Sever do Vouga region represents the biggest area of blueberries production in Portugal. Data from 2008 indicate that this region had approximately 20 ha of blueberry-harvested area, with a production of about 60 tons per year, mostly for exportation to other European countries (Serrado, 2008). In 2016, Portugal produced around 6572 tons of blueberries (INE, 2017).

Although canker, stem blight and dieback symptoms characteristic of *Botryosphaeriaceae* and *Diaporthaceae* have been observed in blueberry plantations in Portugal, very little is known about which species are associated with these symptoms and what is their pathogenic potential. In fact, systematic studies on fungal diseases of blueberry plants in Portugal are scarce.

3. OBJECTIVES

This work aimed to study the fungal biodiversity associated with blueberry plants from plantations in the region of Aveiro (Portugal), with a special focus on the families *Diaporthaceae* and *Botryosphaeriaceae*. The fungi associated to blueberry plants were characterised based on DNA sequence data as well as phenotypic data. Additionally, the pathogenicity of representative isolates of both fungal families was evaluated through artificial inoculation of blueberry plants.

4. MATERIAL AND METHODS

4.1. Sampling

The sampling was carried out in summer 2016 in plantations of highbush blueberry from three regions of Portugal: Aveiro, Ílhavo and Sever do Vouga. Several cultivars were selected randomly, from potted blueberry plants as well as from plants cropped in the field. Samples were taken from symptomatic branches and stems showed twig dieback, browning and blighting tissues, necrotic, drying and death branches. The samples from asymptomatic material were taken from branches and leaves. The symptomatic samples were collected from Aveiro, Sever do Vouga and Ílhavo plantations; the asymptomatic samples were collected only from the Sever do Vouga plantation.

4.2. Fungal Isolation

For symptomatic samples, after peeling the outside bark, small fragments (2-5 mm) of necrotic tissue were cut from the sterilised interface between healthy and diseased branches and stems and plated on half strength potato dextrose agar (½ PDA) (Merck, Germany). For asymptomatic samples, a different procedure was used. Pieces of branches and leaves were surface sterilised in 5 % sodium hypochlorite for 1 minute followed by 96 % ethanol for 1 minute and sterile water for 1 minute. The last step with sterile water was repeated twice. After drying, pieces of branches were cut longitudinally and in the edges; the edges of leaves were also cut and plated on ½ PDA. The plates were incubated at room temperature and checked daily for fungal growth.

Fungal isolates were obtained by transferring mycelial plugs from the margins of the expanding colonies, and placed in ½ PDA or ½ PDA plates supplemented with tetracycline and streptomycin (to prevent bacterial growth). These fungal isolates were kept at 25°C in the dark.

4.3. Purification of cultures

4.3.1. Single conidia

To induce sporulation, 4-day-old mycelium plugs from the different fungal isolates were inoculated on autoclaved (20 min, 121°C, 1 bar) *Pinus pinaster* needles and *Foeniculum vulgare* twigs as described by Santos & Phillips (2009) and Santos et al. (2017). Pine needles and fennel twigs were aseptically placed on 2 % water agar medium (WA) and quarter strength potato dextrose agar medium (¼ PDA). Cultures were secured with Parafilm® and kept at room temperature (20-25°C) for about 2 months, under diffuse daylight. All isolates produced globular, black, white or yellow conidial cirrhus on both pine needles and fennel twigs.

Conidia were taken from the pycnidia, crushed in a drop of sterile water and spread over the surface of 90 mm ¼ PDA plates. This procedure was made with the support of a Nikon stereomicroscope SMZ1500 (Nikon, Japan). After incubating for 24 hours, single germinating spores were carefully transferred with a sterile scalpel into ¼ PDA plates and stored at 25°C in the dark. Spores were also placed in a drop of water on a microscope slide and then mounted in 100 % lactic acid for morphological characters observation. All preparations were observed with a Nikon 80i compound microscope (Nikon, Japan) and photographed with a Nikon Digital Sight DS-Ri1 camera (Nikon, Japan).

4.3.2. Hyphal tip culturing

From samples that could not be induced to sporulate, pieces of mycelium were removed and transferred to a ¼ PDA or WA plates, nutrient poor medium which facilitates slower growth of the fungus and incubated at 25°C in the dark. After 24 hours, a single hyphal tip was cut and transferred to a ¼ PDA plate. After 3 days at 25°C, if they were sufficiently grown, they were transferred for ½ PDA medium and pure cultures were obtained.

4.4. DNA extraction

The total genomic DNA was extracted from 7-days-old cultures according to a modified protocol of Möller (1992). Cultures were incubated on PDA for seven days at 25°C or until enough mycelium development. Microtubes (2 mL) were correctly tagged for each sample and 500 µL of sterile TES buffer (100 Mm Tris, pH 8.0; 10 Mm EDTA, pH 8.0; 2 % SDS) was added to each one. TES buffer stock should be sterile and stored at room temperature. Mycelium was scraped from the petri plate and transferred into the microtubes with TES. The microtubes with mycelia and TES were heated at 100°C for 3 minutes (for lysing the mycelium), before being placed on ice for 10 minutes. After that, 5 µL of proteinase K (20 mg/mL) was added to each microtube and incubated at 65°C for 30 minutes with mixing by inversion. The salt concentration was then increased by adding 140 µL of 5 M NaCl. Plus, 65 µL of 10 % cetyltrimethylammoniumbromide (CTAB) was also added. Microtubes were incubated at 65°C for 30 minutes with occasional swirl. One mL of chloroform:isoamylalcohol (CIA) in a proportion of 24:1 was added, tubes were mixed carefully by inversion for 1 minute and then incubated on ice for 30 minutes. Tubes were centrifuged for 10 minutes at 12000 rpm and 4°C before transferring the supernatant (±800 µL) to a new 1.5 mL microtube. 225 µL of 5 M NH_4OAc was further added to the new tubes, mixed carefully, and then incubated for 30 minutes on ice and centrifuged for 10 minutes at 12000 rpm at 4°C. The supernatant (±900 µL) was removed and transferred to a new 1.5 mL microtube, 500 µL of isopropanol was added and mixed gently. The tubes were then incubated for 30 minutes on ice or placed at -20ºC. Finally, microtubes were centrifuged for 10 minutes at 12000 rpm at 4°C and the supernatant was discarded. This step using isopropanol induced the selective aggregation and precipitation of DNA. The resulting DNA pellet was dried at room temperature and then dissolved with 50 µL of TE buffer (1 M Tris, pH 8.0; 0.5 M EDTA, pH 8.0). Tubes were then stored at -20°C.

4.5. Storage of cultures

For the storage of cultures, four plugs of mycelium were taken from the margin of a 7-day-old colony of the selected isolates and placed into cryotubes (1.5 mL) with 800 µL of 15 % glycerol solution. The cryotubes were kept at room temperature overnight and then stored at -80°C.

4.6. PCR Fingerprinting

BOX-PCR fingerprinting was done according to Alves et al. (2007). The amplification reaction, with a final volume of 25 µL, contained: 15.75 µL of sterile pure water, 6.25 µL of NZYTaq 2xgreen Master Mix (2.5 mM MgCl2; 200 µM dNTPs; 0.2 U/µL DNA polymerase; Nzytech™, Portugal), 2 µL of BOXA1R primer (5'-CTACGGCAAGGCGACGCTGACG-3'; Invitrogen™, USA) (Versalovic et al., 1994) at 10 pmol/µL and 1 µL of DNA template. A PCR reaction with no DNA template was always included as a negative control. After an initial denaturation for 5 minutes at 95°C, 30 cycles were performed at 94°C for 1 minute, followed by annealing at 53°C for 1 minute and extension at 65°C for 8 minutes, with a final elongation step at 65 °C for 16 minutes (Alves et al., 2007). After the amplification, a 1.5 % agarose gel was loaded with 5 µL of sample and the electrophoresis was carried out at 80 V in TAE 1x (40 mM Tris, pH 7.6; 20 mM acetic acid; 1 mM EDTA) during 2h45min. All gels were further visualised under UV light (GELDOC XR+, Bio-Rad, USA) after staining with ethidium bromide for 15-20 minutes and then washed with distillated water for 1 hour.

The fingerprint profiles of all isolates were analysed with GelCompar II software (Applied Maths). Similarity between the profiles was calculated with the Pearson's correlation coefficient. Cluster analysis of similarity matrices was performed by the unweighted pair group method using arithmetic averages (UPGMA).

4.7. DNA sequencing

Five loci (ITS, *tef1-α*, *his*, *tub* and *cal*) were amplified and sequenced. Primers ITS5 and NL4/LR3 were used to amplify the ITS region (White et al, 1990; Vilgalys & Hester, 1990). PCR conditions were as follows: initial denaturation for 5 minutes at 95°C, 30 cycles were performed at 94°C for 30 seconds, followed by the annealing step at 50 °C for 30 seconds and extension at 72°C for 1.5 minutes, with a final elongation step at 72°C for 10 minutes.

Tef1-α gene was amplified using 3 primer sets depending on the isolates: EF-688F and EF-1251R; EF-1251R and EF-728F and EF-688F and EF-986R (Alves et al., 2008; Carbone & Kohn, 1999). The primer sets T1 and Bt2b, Bt2b and Bt2a were used to sequence part of the *tub* gene (Glass & Donaldson 1995; O'Donnell & Cigelnik 1997). The primer set CYLH3F and H3-1bR was used to amplify the *his* gene (Crous et al., 2004; Glass & Donaldson 1995). To amplify part of the *cal* gene, CAL-228F and CAL-737R primers were used (Carbone & Kohn 1999). The DNA sequence of the primers used are described in Table 2.

All PCR reaction mixtures, with a final volume of 25 µl, were composed of 15.75 µL of sterile pure water, 6.25 µL of NZYTaq 2xgreen Master Mix, 1 µL of each primer at 10 pmol/µL and 1 µL of DNA template. A negative control was always included.

PCR reactions were performed in a BIO-RAD C1000 touch^{TM} Thermal Cycler (USA). To amplify *tef1-α*, *cal, his* and *tub* region the denaturation step ran for 5 minutes at 95°C, followed by 30 PCR cycles at 94°C for 30 seconds, annealing at 52, 53 and 60ºC for 30 seconds (for *tef1-α*/*tub*, *cal* and *his*, respectively), and extension at 72°C for 1 minute with a final elongation step at 72°C for 10 minutes. Depending on the isolates, 35 PCR cycles for *tef1-α* gene amplification were performed.

PCR products were separated by electrophoresis at 80 V for 60 minutes (ITS, *tef1-α*, *his*, *tub* and *cal*) in an 1.5 % agarose gel in 1x TAE (40 mM Tris, pH 7.6; 20 mM Acetic acid; 1 mM EDTA). For each electrophoresis, DNA ladder (GeneRuler DNA Ladder Mix 0.5 µg/µL, 50 µg, Thermo Scientific ^{TM}, USA) was loaded in the gel. The gels were visualised under UV light (GELDOC XR+, Bio-

Rad, USA) after staining with ethidium bromide for 5 minutes and washed in water for 20 minutes.

Amplicons were purified using the NZYGelPure Kit (Nzytech™, Portugal) according to manufacturer's instructions. Both strands of the amplicons were sequenced by GATC Biotech (Germany).

Table 2 – Primers DNA sequences used in the study.

Locus	Primer	Primer DNA sequence (5'-3')
ITS	ITS5	GGAAGTAAAAGTCGTAACAAGG
	NL4/LR3	GGTCCGTGTTTCAAGACGG
tef1-α	EF-688F	CGGTCACTTGATCTACAAGTGC
	EF-1251R	CCTCGAACTCACCAGTACCG
	EF-728F	CATCGAGAAGTTCGAGAAGG
	EF-986R	TACTTGAAGGAACCCTTACC
cal	CAL-228F	GAGTTCAAGGAGGCCTTCTCCC
	CAL-737R	CATCTTTCTGGCCATCATGG
his	CYLH3F	AGGTCCACTGGTGGCAAG
	H3-1Br	GCGGGCGCGAGCTGGATGTCCTT
tub	T1	AACATGCGTGAGATTGTAAGT
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC
	Bt2a	GGTAACCAAATCGGTGCTGCTTT
MAT	MAT1-1F	GCAAMIGTKTIKACTCACA
	MAT1-1R	GTCTMTGACCARGACCATG
	MAT1-2F	GCCCKCCYAAYCCATTCATC
	MAT1-2R	TTGACYTCGAAGACTTGCGTG

4.8. Phylogenetic analysis

The nucleotide sequences were read and edited with FinchTV v.1.4 (Geospiza Inc. http://www.geospiza.com/finchtv). A primary identification was done using Standard Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/), based on *Query cover* and *Identity* parameters. Sequences of our isolates were aligned with all available *Botryosphaeria*, *Neofusicoccum* and *Diaporthe* sequences from GenBank.

The alignment was made with CLUSTALX v.2.0 using the following parameters: pairwise alignment (gap opening = 10, gap extension = 0.1) and multiple alignment (gap opening = 10, gap extension = 0.2, transition weight = 0.5, delay divergent sequences = 25 %). The alignments were checked and edited manually using BioEdit Sequence Alignment Editor. MEGA7 v. 7.0 (http://www.megasoftware.net) was used to determine the best model of the DNA evolution to be used for the Maximum Likelihood (ML) analyses (Kumar et al., 2016). Nearest-Neighbour-Interchange (NNI) was used as the heuristic method for tree inference with 1000 bootstrap replicates. No outgroup species was used for *Botryosphaeria* and *Neofusicoccum* phylogenies. *Diaporthella corylina* (CBS 121124) was chosen as outgroup for the phylogenetic analysis of *Diaporthe* (Santos et al., 2017).

4.9. Mating type assay

The mating strategy of *Diaporthe* isolates was determined by a PCR-based mating type assay, using the set of primers DiaMAT1F/DiaMAT1R for MAT1-1 and DiaMAT2F/DiaMAT2R for MAT1-2 developed by Santos et al. (2010). The DNA sequence of the primers are described in Table 2. Part of the MAT1-1 and MAT1-2 genes was amplified using: 1µL of DNA, 4 µL of each MAT1-1F and MAT1-1R primer at 40 pmol and 1 µL of each MAT1-2F and MAT1-2R primer at 10 pmol; 1.25 µL of dimethyl sulfoxide (DMSO) at 1 % and 6.25 µL of NZYTaq 2x Green Master Mix (2.5 mM MgCl2; 200 µM dNTPs; 0.2 U/µL DNA polymerase; Nzytech™, Portugal).

The PCR cycling conditions were as described by Santos et al. (2010): 5 minutes at 95 °C, followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 50°C (for MAT1-1F and MAT1-1R primers) and 56°C (for MAT1-2F and MAT1-2R primers) and 1 minute at 72°C, with a final step of 10 minutes at 72°C. All PCR products were visualised under ultraviolet light in a 2 % agarose gel stained with ethidium bromide.

4.10. Pathogenicity trials

One representative isolate of each *Diaporthe*, *Neofusicoccum* and *Botryosphaeria* species identified (M89 – *Botryosphaeria dothidea*, M207 – *Neofusicoccum australe*, M85 – *N. eucalyptorum*, M97 – *N. parvum*, M65 – *Diaporthe rudis*, M101 – *D. eres*, M118 – *D. foeniculina*, M116 – *Diaporthe* sp. 1, M164 – *Diaporthe* sp. 2, CAA762 – *Diaporthe* sp. 3) was used for pathogenicity assays.

The pathogenicity tests were performed on healthy branches and stems of potted *Vaccinium corymbosum* plants (cultivar *Bluecrop*). Nine plants were inoculated with each isolate. For inoculation, a piece of the bark tissue was cut using a sterile scalpel exposing the cambium. A 5-mm-diameter mycelial plug was taken from 7-day-old cultures on ½ PDA and placed in the wounded area with the mycelium in contact with the plant tissue. The inoculation region was sealed with Parafilm® to avoid rapid dehydration. Plugs of uninoculated ½ PDA were used as negative controls. The plants were maintained at room temperature for 2 months after which the size necrotic lesions was measured. Symptoms were checked regularly and registed. A one-way analysis of variance (ANOVA) followed by a Student test was used to determine the significance of differences between means. Analyses were done with JMP®8.0.1 (SAS Institute Inc., NC, USA).

4.11. Temperature growth studies

The putative new species identified through phylogenetic analysis, were inoculated on ½ PDA plates and incubated at 25°C for 7 days or until the colony reached the edges of the plate. From these cultures, a 5 mm plug for each isolate was placed in the centre of PDA plates. Three replicate plates per isolate were incubated at 5, 10, 20, 25, 30, 35 and 40°C. The effect of temperature on colony growth was examined daily for 6 days and determined by measuring diameter in two perpendicular directions in each replicate.

5. RESULTS

5.1. Sampling and Fungal Isolation

From 19 blueberry plants collected, 49 samples (38 symptomatic and 11 asymptomatic) were selected for fungal isolation. From these samples, 222 isolates (Table 3) were obtained of which 202 from symptomatic plants (35 obtained from Aveiro; 6 from Ílhavo and 161 from Sever do Vouga) and 20 from asymptomatic plant material (from Sever do Vouga). These 222 isolates include 35 (identified in Table 3 with the acronym CAA) that were obtained previously (Amaral, 2016). With the purpose to obtain pure cultures, hyphal tip culturing and single conidia procedures were required.

Table 3 – Fungal isolates obtained from blueberry samples.

Isolate	Sampling date	Sampling location	Cultivar	Symptoms
CAA761	August 2015	Sever do Vouga	Duke	branch canker
CAA762	August 2015	Sever do Vouga	Duke	branch canker
CAA763	August 2015	Sever do Vouga	Duke	branch canker
CAA764	August 2015	Sever do Vouga	Duke	branch canker
CAA766	October 2015	Sever do Vouga	Ozarkblue	asymptomatic branch
CAA767	October 2015	Sever do Vouga	Ozarkblue	asymptomatic branch
CAA768	October 2015	Sever do Vouga	Ozarkblue	asymptomatic branch
CAA769	October 2015	Sever do Vouga	Ozarkblue	asymptomatic branch
CAA770	October 2015	Sever do Vouga	Ozarkblue	asymptomatic branch
CAA771	October 2015	Sever do Vouga	Ozarkblue	asymptomatic root
CAA772	October 2015	Sever do Vouga	Ozarkblue	branch canker
CAA773	October 2015	Sever do Vouga	Ozarkblue	branch canker
CAA774	October 2015	Sever do Vouga	Draper	asymptomatic root
CAA775	October 2015	Sever do Vouga	Draper	asymptomatic root
CAA776	October 2015	Sever do Vouga	Draper	branch canker
CAA777	October 2015	Sever do Vouga	Draper	branch canker
CAA778	October 2015	Sever do Vouga	Draper	branch canker
CAA779	October 2015	Sever do Vouga	Draper	asymptomatic branch
CAA780	October 2015	Sever do Vouga	Ozarkblue	stem canker
CAA781	October 2015	Sever do Vouga	Ozarkblue	stem canker
CAA782	October 2015	Sever do Vouga	Ozarkblue	stem canker
CAA783	October 2015	Sever do Vouga	Ozarkblue	stem canker
CAA784	October 2015	Sever do Vouga	Ozarkblue	stem canker
CAA785	October 2015	Sever do Vouga	Ozarkblue	stem canker
CAA786	October 2015	Sever do Vouga	Ozarkblue	stem canker
CAA787	October 2015	Sever do Vouga	Ozarkblue	branch canker
CAA788	October 2015	Sever do Vouga	Ozarkblue	branch canker
CAA789	October 2015	Sever do Vouga	Ozarkblue	stem canker
CAA790	October 2015	Sever do Vouga	Ozarkblue	stem canker
CAA791	October 2015	Sever do Vouga	Ozarkblue	stem canker
CAA792	October 2015	Sever do Vouga	Ozarkblue	stem canker
CAA793	October 2015	Sever do Vouga	Ozarkblue	branch canker

CAA794	October 2015	Sever do Vouga	Ozarkblue	stem canker
CAA795	October 2015	Sever do Vouga	Ozarkblue	stem canker
CAA796	October 2015	Sever do Vouga	Ozarkblue	asymptomatic root
M2	June 2016	Aveiro	Duke	branch canker
M3	June 2016	Aveiro	Duke	dieback
M4	June 2016	Aveiro	Duke	dieback
M5	June 2016	Aveiro	Duke	dieback
M6	June 2016	Aveiro	Duke	dieback
M8	June 2016	Aveiro	Duke	dieback
M9	June 2016	Aveiro	Duke	dead branch
M10	June 2016	Aveiro	Duke	dead branch
M12	June 2016	Aveiro	Duke	dead branch
M13	June 2016	Aveiro	Duke	dead branch
M14	June 2016	Aveiro	Duke	dead branch
M15	June 2016	Aveiro	Duke	dead branch
M17	June 2016	Aveiro	Duke	dead branch
M18	June 2016	Aveiro	Duke	dead branch
M19	June 2016	Aveiro	Duke	dead branch
M20	June 2016	Aveiro	Duke	dead branch
M22	June 2016	Aveiro	Duke	dead branch
M23	June 2016	Aveiro	Duke	dead branch
M24	June 2016	Aveiro	Duke	dead branch
M25	June 2016	Aveiro	Duke	dead branch
M26	June 2016	Aveiro	Duke	dead branch
M27	June 2016	Aveiro	Duke	dead branch
M30	June 2016	Aveiro	Duke	dead branch
M31	June 2016	Aveiro	Duke	dead branch
M32	June 2016	Aveiro	Duke	dead branch
M33	June 2016	Aveiro	Duke	dead branch
M34	June 2016	Aveiro	Duke	dead branch
M35	June 2016	Aveiro	Duke	dead branch
M36	June 2016	Aveiro	Duke	dead branch
M37	June 2016	Aveiro	Duke	dead branch
M39	June 2016	Sever do Vouga	Unknown	asymptomatic branch
M43	June 2016	Sever do Vouga	Unknown	asymptomatic branch
M47	June 2016	Sever do Vouga	Unknown	yellow and red leaves
M50	June 2016	Sever do Vouga	Unknown	yellow and red leaves
M51	June 2016	Sever do Vouga	Unknown	yellow and red leaves
M55	June 2016	Sever do Vouga	Unknown	asymptomatic branch
M56	June 2016	Sever do Vouga	Unknown	yellow and red leaves
M58	June 2016	Sever do Vouga	Unknown	yellow and red leaves
M59	June 2016	Sever do Vouga	Unknown	yellow and red leaves
M65	June 2016	Sever do Vouga	Unknown	yellow and red leaves
M68	June 2016	Ílhavo	Duke	branch canker
M69	June 2016	Ílhavo	Duke	branch canker
M71	June 2016	Ílhavo	Duke	branch canker
M72	June 2016	Ílhavo	Duke	branch canker
M73	June 2016	Ílhavo	Duke	branch canker
M85	June 2016	Ílhavo	Duke	dead branch
M89	July 2016	Sever do Vouga	Unknown	dead branch
M90	July 2016	Sever do Vouga	Unknown	dead branch
M95	July 2016	Sever do Vouga	Unknown	dieback
M96	July 2016	Sever do Vouga	Unknown	dieback
M97	July 2016	Sever do Vouga	Unknown	dead branch
M99	July 2016	Sever do Vouga	Unknown	dead branch

M100	July 2016	Sever do Vouga	Unknown	dead branch
M101	July 2016	Sever do Vouga	Unknown	dead branch
M102	July 2016	Sever do Vouga	Unknown	dead branch
M103	July 2016	Sever do Vouga	Unknown	dead branch
M104	July 2016	Sever do Vouga	Unknown	dead branch
M106	July 2016	Sever do Vouga	Unknown	dead branch
M107	July 2016	Sever do Vouga	Unknown	dead branch
M108	July 2016	Sever do Vouga	Unknown	dead branch
M111	July 2016	Sever do Vouga	Unknown	dead branch
M112	July 2016	Sever do Vouga	Unknown	dead branch
M113	July 2016	Sever do Vouga	Unknown	dead branch
M114	July 2016	Sever do Vouga	Unknown	dead branch
M115	July 2016	Sever do Vouga	Unknown	dead branch
M116	July 2016	Sever do Vouga	Unknown	dead branch
M117	July 2016	Sever do Vouga	Unknown	asymptomatic branch
M118	July 2016	Sever do Vouga	Unknown	asymptomatic branch
M121	July 2016	Sever do Vouga	Unknown	dead branch
M122	July 2016	Sever do Vouga	Unknown	dead branch
M124	July 2016	Sever do Vouga	Unknown	dead branch
M126	July 2016	Sever do Vouga	Unknown	dead branch
M129	July 2016	Sever do Vouga	Unknown	dead branch
M130	July 2016	Sever do Vouga	Unknown	dead branch
M133	July 2016	Sever do Vouga	Unknown	dieback
M134	July 2016	Sever do Vouga	Unknown	dieback
M135	July 2016	Sever do Vouga	Unknown	dieback
M136	July 2016	Sever do Vouga	Unknown	dieback
M137	July 2016	Sever do Vouga	Unknown	dieback
M138	July 2016	Sever do Vouga	Unknown	dieback
M139	July 2016	Sever do Vouga	Unknown	dieback
M140	July 2016	Sever do Vouga	Unknown	dieback
M141	July 2016	Sever do Vouga	Unknown	dieback
M142	July 2016	Sever do Vouga	Unknown	dieback
M144	July 2016	Sever do Vouga	Unknown	dieback
M145	July 2016	Sever do Vouga	Unknown	dieback
M146	July 2016	Sever do Vouga	Unknown	dieback
M147	July 2016	Sever do Vouga	Unknown	dieback
M148	July 2016	Sever do Vouga	Unknown	dieback
M149	July 2016	Sever do Vouga	Unknown	dieback
M150	July 2016	Sever do Vouga	Unknown	dieback
M155	July 2016	Sever do Vouga	Unknown	dieback
M156	July 2016	Sever do Vouga	Unknown	dieback
M157	July 2016	Sever do Vouga	Unknown	dieback
M158	July 2016	Sever do Vouga	Unknown	dieback
M159	July 2016	Sever do Vouga	Unknown	dieback
M160	July 2016	Sever do Vouga	Unknown	dieback
M161	July 2016	Sever do Vouga	Unknown	dieback
M162	July 2016	Sever do Vouga	Unknown	dieback
M163	July 2016	Sever do Vouga	Unknown	dieback
M164	July 2016	Sever do Vouga	Unknown	dieback
M165	July 2016	Sever do Vouga	Unknown	dieback
M166	July 2016	Sever do Vouga	Unknown	dieback
M168	July 2016	Sever do Vouga	Unknown	dieback
M169	July 2016	Sever do Vouga	Unknown	dead branch
M171	July 2016	Sever do Vouga	Unknown	dead branch
M172	July 2016	Sever do Vouga	Unknown	dead branch

M175	July 2016	Sever do Vouga	Unknown	dead branch
M176	July 2016	Sever do Vouga	Unknown	dead branch
M177	July 2016	Sever do Vouga	Unknown	dead branch
M180	July 2016	Sever do Vouga	Unknown	dead branch
M182	July 2016	Sever do Vouga	Unknown	dead branch
M184	July 2016	Sever do Vouga	Unknown	dead branch
M185	July 2016	Sever do Vouga	Unknown	dead branch
M186	July 2016	Sever do Vouga	Unknown	dead branch
M189	July 2016	Sever do Vouga	Unknown	dead branch
M190	July 2016	Sever do Vouga	Unknown	dead branch
M191	July 2016	Sever do Vouga	Unknown	dead branch
M197	July 2016	Sever do Vouga	Unknown	dead branch
M198	July 2016	Sever do Vouga	Unknown	dead branch
M201	July 2016	Sever do Vouga	Unknown	dead branch
M203	July 2016	Sever do Vouga	Unknown	dead branch
M204	July 2016	Sever do Vouga	Unknown	dead branch
M205	July 2016	Sever do Vouga	Unknown	dead branch
M207	July 2016	Sever do Vouga	Unknown	dead branch
M209	July 2016	Sever do Vouga	Unknown	dead branch
M210	July 2016	Sever do Vouga	Unknown	dead branch
M214	July 2016	Sever do Vouga	Unknown	dieback
M219	July 2016	Sever do Vouga	Unknown	dieback
M220	July 2016	Sever do Vouga	Unknown	dieback
M222	July 2016	Sever do Vouga	Unknown	dieback
M223	July 2016	Sever do Vouga	Unknown	dieback
M224	July 2016	Sever do Vouga	Unknown	dieback
M225	July 2016	Sever do Vouga	Unknown	dieback
M226	July 2016	Sever do Vouga	Unknown	dieback
M227	July 2016	Sever do Vouga	Unknown	dead branch
M228	July 2016	Sever do Vouga	Unknown	dead branch
M229	July 2016	Sever do Vouga	Unknown	dead branch
M230	July 2016	Sever do Vouga	Unknown	dead branch
M231	July 2016	Sever do Vouga	Unknown	dead branch
M232	July 2016	Sever do Vouga	Unknown	dead branch
M233	July 2016	Sever do Vouga	Unknown	dead branch
M234	July 2016	Sever do Vouga	Unknown	dead branch
M235	July 2016	Sever do Vouga	Unknown	dead branch
M236	July 2016	Sever do Vouga	Unknown	dead branch
M237	July 2016	Sever do Vouga	Unknown	dead branch
M240	July 2016	Sever do Vouga	Unknown	dead branch
M241	July 2016	Sever do Vouga	Unknown	dead branch
M243	July 2016	Sever do Vouga	Unknown	dead branch
M245	July 2016	Sever do Vouga	Unknown	dead branch
M246	July 2016	Sever do Vouga	Unknown	dead branch
M247	July 2016	Sever do Vouga	Unknown	dead branch
M248	July 2016	Sever do Vouga	Unknown	dead branch
M251	July 2016	Sever do Vouga	Unknown	dead branch
M252	July 2016	Sever do Vouga	Unknown	dead branch
M253	July 2016	Sever do Vouga	Unknown	dieback
M255	July 2016	Sever do Vouga	Unknown	dieback
M256	July 2016	Sever do Vouga	Unknown	Dieback
M259	July 2016	Sever do Vouga	Unknown	dieback
M261	July 2016	Sever do Vouga	Unknown	dieback
M264	July 2016	Sever do Vouga	Unknown	dieback
M265	July 2016	Sever do Vouga	Unknown	dieback

M266	July 2016	Sever do Vouga	Unknown	Dieback
M267	July 2016	Sever do Vouga	Unknown	dieback
M270	July 2016	Sever do Vouga	Unknown	dieback
M271	July 2016	Sever do Vouga	Unknown	dieback
M276	July 2016	Sever do Vouga	Unknown	dieback
M291	July 2016	Sever do Vouga	Unknown	stem canker
M295	July 2016	Sever do Vouga	Unknown	stem canker
M298	July 2016	Sever do Vouga	Unknown	stem canker
M304	July 2016	Sever do Vouga	Unknown	branch canker
M305	July 2016	Sever do Vouga	Unknown	branch canker
M306	July 2016	Sever do Vouga	Unknown	branch canker
M308	July 2016	Sever do Vouga	Unknown	branch canker
M313	July 2016	Sever do Vouga	Unknown	branch canker
M316	July 2016	Sever do Vouga	Unknown	branch canker
M317	July 2016	Sever do Vouga	Unknown	branch canker
M319	July 2016	Sever do Vouga	Unknown	asymptomatic branch
M320	July 2016	Sever do Vouga	Unknown	asymptomatic branch
M323	July 2016	Sever do Vouga	Unknown	asymptomatic branch
M328	July 2016	Sever do Vouga	Unknown	asymptomatic branch
M331	July 2016	Sever do Vouga	Unknown	asymptomatic branch
M332	July 2016	Sever do Vouga	Unknown	asymptomatic branch
M333	July 2016	Sever do Vouga	Unknown	asymptomatic branch
M334	July 2016	Sever do Vouga	Unknown	asymptomatic branch
M335	July 2016	Sever do Vouga	Unknown	asymptomatic branch
M336	July 2016	Sever do Vouga	Unknown	asymptomatic branch

5.2. PCR Fingerprinting and ITS identification

A total of 222 isolates were characterised by BOX-PCR fingerprinting to evaluate their overall genetic diversity. Based on the similarity of the profiles, only 81 representative isolates were selected for ITS amplification and further sequencing. A primary identification was done using Standard Nucleotide BLAST (Table 4).

Table 4 - Isolate identification using ITS sequences and BLASTn against GenBank database.

Isolate	ITS identification	Identity (%)
CAA761	*Stemphylium vesicarium*	100 %
CAA762	*Diaporthe* sp. 3	99 %
CAA763	*Diaporthe* sp. 3	99 %
CAA764	*Peyronellaea glomerata*	99 %
CAA766	*Alternaria tenusissima*	100 %
CAA767	*Botryosphaeria dothidea*	100 %
CAA768	*Neofusicoccum australe*	100 %
CAA771	*Trichoderma hamatum*	100 %
CAA773	*Botryosphaeria dothidea*	99 %
CAA774	*Trichoderma paraviridescens*	99 %

CAA775	*Trichoderma paraviridescens*	100 %
CAA776	*Colletotrichum gloesporioides*	100 %
CAA777	*Diaporte rudis*	100 %
CAA779	*Alternaria alternata*	100 %
CAA784	*Botryosphaeria dothidea*	100 %
CAA785	*Botryosphaeria dothidea*	100 %
CAA788	*Botryosphaeria dothidea*	100 %
CAA789	*Diaporthe rudis*	100 %
CAA790	*Diaporthe rudis*	100 %
CAA796	*Trichoderma harzianum*	100 %
M3	*Phlebiopsis* sp.	100 %
M8	*Stemphylium vesicarium*	100 %
M9	*Alternaria alternata*	100 %
M15	*Diaporthe rudis*	100 %
M17	*Diaporthe foeniculina*	99 %
M18	*Diaporthe foeniculina*	99 %
M19	*Diaporthe foeniculina*	100 %
M20	*Diaporthe foeniculina*	100 %
M22	*Neofusicoccum parvum*	100 %
M23	*Neofusicoccum parvum*	100 %
M35	*Colletotrichum gloesporioides*	100 %
M37	*Colletotrichum gloesporioides*	100 %
M39	*Paraphaeosphaeria* sp.	100 %
M43	*Diaporthe* sp. 2	100 %
M51	*Colletotrichum* sp.	100 %
M55	*Alternaria* sp.	100 %
M56	*Stemphylium solani*	100 %
M65	*Diaporthe rudis*	100 %
M69	*Alternaria* sp.	100 %
M71	*Alternaria* sp.	100 %
M85	*Neofusicoccum eucalyptorum*	100 %
M89	*Botryosphaeria dothidea*	100 %
M95	*Neofusicoccum australe*	100 %
M97	*Neofusicoccum parvum*	100 %
M99	*Diaporthe* sp. 1	98 %
M101	*Diaporthe eres*	100 %
M116	*Diaporthe* sp. 1	98 %
M118	*Diaporthe foeniculina*	99 %
M126	*Botryosphaeria dothidea*	100 %
M134	*Diaporthe eres*	100 %
M146	*Neurospora* sp.	99 %
M155	*Diaporthe* sp. 2	100 %
M156	*Diaporthe* sp. 2	99 %
M162	*Diaporthe* sp. 2	100 %
M164	*Diaporthe* sp. 2	100 %
M165	*Neofusicoccum parvum*	100 %
M171	*Botryosphaeria dothidea*	100 %
M189	*Neofusicoccum parvum*	100 %

M198	*Neofusicoccum australe*	100 %
M207	*Neofusicoccum australe*	100 %
M210	*Botryosphaeriae dothidea*	100 %
M219	*Phlebia acerina*	100 %
M222	*Neofusicoccum eucalyptorum*	100 %
M229	*Neofusicoccum parvum*	100 %
M233	*Neofusicoccum parvum*	100 %
M237	*Neofusicoccum parvum*	100 %
M240	*Neofusicoccum parvum*	100 %
M241	*Neofusicoccum eucapyptorum*	100 %
M246	*Neofusicoccum eucalyptorum*	100 %
M253	*Neofusicoccum parvum*	100 %
M265	*Pestalotiopsis* sp.	99 %
M291	*Diaporthe* sp. 2	99 %
M295	*Diaporthe* sp. 2	99 %
M298	*Diaporthe* sp. 2	99 %
M304	*Botryosphaeria dothidea*	100 %
M308	*Pestalotiopsis* sp.	100 %
M317	*Neofusicoccum parvum*	100 %
M323	*Neofusicoccum australe*	100 %
M328	*Neofusicoccum parvum*	100 %
M333	*Colletotrichum* sp.	99 %
M336	*Neofusicoccum parvum*	100 %

From this primary identification, our isolates were grouped in 4 different groups. Genus *Diaporthe* (24 %), genus *Neofusicoccum* (35 %), genus *Botryosphaeria* (18 %) and other genera (23 %) including *Neurospora*, *Colletotrichum*, *Stemphylium*, *Pestalotiopsis*, *Phlebia, Phlebiopsis*, *Alternaria*, *Trichoderma*, *Peyronellaea* and *Paraphaeosphaeria*. *Botryosphaeria dothidea* was the only species found from the genus and with an abundance of 100 %. Among *Neofusicoccum* isolates, *N. parvum* showed to be the most common species found (73 %) followed by *N. australe* and *N. eucalyptorum* with an abundance of 15 % and 12 % respectively. *Diaporthe foeniculina* (11 %), *D. rudis* (15 %), *D. eres* (27 %), *Diaporthe* sp. 1 (11 %), *Diaporthe* sp. 2 (32 %) and *Diaporthe* sp. 3 (4 %) were also found. Once it was our interest to study only *Botryosphaeriaceae* and *Diaporthe* species, all remaining genera were not used for further molecular characterisation.

Although the sequencing of amplified ITS is advantageous to discriminate the isolates at genus level and to give a hint about the species, the identification is not 100 % reliable. For that, partial sequence of the *tef1-α* gene was needed for an

accurate identification of the *Neofusicoccum* and *Botryosphaeria* isolates. For *Diaporthe*, partial sequences of *tef1-α, tub, cal* and *his* were required for a further identification of the isolates.

5.3. Phylogenetic analysis

All available ITS sequences for *Diaporthe*, *Neofusicoccum* and *Botryosphaeria* species were included in an initial phylogenetic analysis to find close relatives of our isolates. Identity of the isolates and GenBank accession numbers of sequences used in phylogenetic analyses are described in Table 5 for *Botryosphaeria*, Table 6 for *Neofusicoccum* and Table 7 for *Diaporthe*. Multi-loci analysis combining the 2 loci ITS and *tef1-α* and the 5 loci ITS, *tef1-α*, *tub*, *cal* and *his* were also conducted.

Table 5 - Species and sequences database accession numbers used in this study for genus *Botryosphaeria*. Ex-type isolates are given in **bold**. (Note: sequence marked with the symbol — is not available in the GeneBank)

Species	Culture	Host	Location	ITS	*tef1-α*
Botryosphaeria agaves	**CBS 133992**	*Agave* sp.	Thailand	JX646791	JX646856
	MFLUCC 10-0051	*Agave* sp.	Thailand	JX646790	JX646855
B. avasmontanum	**CMW 25413**	*Pinus* sp.	Namibia	KF766167	—
	CBS 121769	*Acacia mellifera*	Namibia	EU101303	EU101348
B. corticis	**CBS 119047**	*Vaccinium corymbosum*	USA	DQ299245	EU017539
	CBS 119048	*V. corymbosum*	USA	DQ299246	EU017540
B. dothidea	**CBS 115476**	*Prunus* sp.	Switzerland	AY236949	AY236898
	CBS 110302	*Vitis vinifera*	Portugal	AY259092	AY573218
B. fabicerniana	**CMW 27094**	*Eucalyptus* sp.	China	HQ332197	HQ332213
	CMW27108	*Eucalyptus* sp.	China	HQ332200	HQ332216
B. fusispora	**MFLUCC 10-0098**	*Entada* sp.	Thailand	JX646789	JX646854
B. kuwatsukai	**CBS 135219**	*Malus domestica*	China	KJ433388	KJ433410
	L5P5	*Pyrus* sp.	China	KJ433395	KJ433417
B. minutispermatia	**GZCC 16-0013**	Dead wood	China	KX447675	KX447678
	GZCC 16-0014	Dead wood	China	KX447676	KX447679
B. ramosa	**CBS 122069**	*E. camaldulensis*	Australia	EU144055	EU144070
B. rosaceae	**CGMCC3.18007**	*Malus* sp.	China	KX197074	KX197094
	CGMCC3.18008	*Amygdalus* sp.	China	KX197075	KX197095
B. scharifii	**CBS 124703**	*Mangifera indica*	Iran	JQ772020	JQ772057
	IRAN1543C	*M. indica*	Iran	JQ772019	JQ772056
B. sinensia	CGMCC3.17723	*Morus* sp.	China	KT343254	KU221233
	CGMCC3.17724	*Juglans regia*	China	KT343256	KU221234
	CFCC 82346	*Juglans regia*	China	KT343257	KU221235

Table 6 - Species and sequences database accession numbers used in this study for genus *Neofusicoccum*. Ex-type isolates are given in **bold**. (Note: sequences marked with the symbol — are not available in the GeneBank)

Species	Culture	Host	Location	ITS	*tef1-α*
Neofusicoccum andinum	**CBS 117453**	*Eucalyptus* sp.	Venezuela	GU251155	GU251287
	CBS 117452	*Eucalyptus* sp.	Venezuela	DQ306263	DQ306264
N. arbuti	**CBS 116131**	*Arbutus menziesii*	USA	AY819720	KF531792
	CBS 117090	*Arbutus menziesii*	USA	AY819724	KF531791
N. australe	**CMW6837**	*Acacia sp*	Australia	AY339262	AY339270
	CMW6853	*Sequoiadendron giganteum*	Australia	AY339263	AY339271
N. batangarum	**CBS 124924**	*Terminalia catappa*	Cameroon	FJ900607	FJ900653
	CBS 124923	*Terminalia catappa*	Cameroon	FJ900608	FJ900654
N. brasiliense	**CMM1338**	*Mangifera indica*	Brazil	JX513630	JX513610
	CMM1285	*Mangifera indica*	Brazil	JX513628	JX513608
N. buxi	**CBS 116.75**	*Buxus sempervirens*	France	KX464165	KX464678
N. corticosae	**CBS 120081**	*Eucalyptus corticosa*	Australia	DQ923533	—
	CBS 118099	*E. camaldulensis*	Australia	KX464168	KX464681
N. cordaticola	**CBS 123634**	*Syzygium cordatum*	South Africa	EU821898	EU821868
	CBS 123635	*Syzygium cordatum*	South Africa	EU821903	EU821873
N. cryptoaustrale	**CMW23785**	*Eucalyptus* sp.	South Africa	FJ752742	FJ752713
	CMW20738	*Eucalyptus citriodora*	South Africa	FJ752740	FJ752710
N. eucalypticola	**CBS 115679**	*Eucalyptus grandis*	Australia	AY615141	AY615133
	CBS 115766	*Eucalyptus rossi*	Australia	AY615143	AY615135
N. eucalyptorum	**CBS 115791**	*Eucalyptus grandis*	South Africa	AF283686	AY236891
	CMW10126	*Eucalyptus gandis*	South Africa	AF 283687	AY236892
N. grevilleae	CBS 129518	*Grevillea aurea*	Australia	JF951137	—
N. hellenicum	**CERC1947**	*Pistacia vera*	Greece	KP217053	KP217061
	CERC1948	*Pistacia vera*	Greece	KP217054	KP217062
N. kwambonambiense	**CBS 123639**	*Syzygium cordatum*	South Africa	EU821900	EU821870
	CBS 123641	*Syzygium cordatum*	South Africa	EU821919	EU821889
N. luteum	CBS 110299	*Vitis vinifera*	Portugal	AY259091	AY573217
	CBS 110497	*Vitis vinifera*	Portugal	EU673311	EU673277
N. lumnitzerae	**CMW41613**	*Lumnitzera racemosa*	South Africa	KU587958	KU587948
N. macroclavatum	**CBS 118223**	*Eucalyptus globulus*	Australia	DQ093196	DQ093217
	WAC12445	*Eucalyptus globulus*	Australia	DQ093197	DQ093218
N. mangroviorum	**CMW41364**	Mangrove trees	South Africa	KU587959	KU587949
N. mediterraneum	**CBS 121718**	*Eucalyptus sp.*	Greece	GU251176	GU251308
	CBS 121558	*Vitis vinifera*	USA	GU799463	GU799462
N. mangiferae	**CBS 118531**	*Magnifera indica*	Australia	AY615185	DQ093221

	CBS 118532	*Magnifera indica*	Australia	AY615186	DQ093220
N. nonquaesitum	**CBS 126655**	*Umbellularia californica*	USA	GU251163	GU251295
	PD301	*Vaccinium corymbosum*	Chile	GU251164	GU251296
N. occulatum	**CBS 128008**	*Eucalyptus grandis*	Australia	EU301030	EU339509
	MUCC286	*Eucalyptus pellita*	Australia	EU736947	EU339511
N. parvum	**CMW9081**	*Populus nigra*	New Zealand	AY236943	AY236888
	CBS 110301	*Vitis vinifera*	Portugal	AY259098	AY573221
N. pennatisporum	**MUCC510**	*Allocasuarina fraseriana*	Australia	EF591925	EF591976
N. pistcaciae	**CBS 595.76**	*Pistacia vera*	Greece	KX464163	KX464676
N. pistaciarum	**CBS 113083**	*Pistacia vera*	USA	KX464186	KX464712
N. protearum	MUCC497	*Santalum acuminatum*	Australia	EF591912	EF591965
N. ribis	**CBS 115475**	*Ribes sp.*	USA	AY236935	AY236877
	CBS 121.26	*Ribes sp.*	USA	AF241177	AY236879
N. stellenboschiana	**CBS 110864**	*Vitis vinifera*	South Africa	KX464225	KX464758
N. umdonicola	**CBS 123645**	*Syzygium cordatum*	South Africa	EU821904	EU821874
	CBS 123646	*Syzygium cordatum*	South Africa	EU821905	EU821875
N. ursorum	**CBS 122811**	*Eucalyptus sp.*	South Africa	FJ752746	FJ752709
	CBS 122812	*Eucalyptus arboretum*	South Africa	KX464227	KX464760
N. viticlavatum	**CBS 112878**	*Vitis vinifera*	South Africa	AY343381	AY343342
N. vitifusiforme	5H022	*Juglans regia*	California	KF778869	KF779059
	B8	*Vitis vinifera*	Italy	KC469638	KX505897

Table 7 - Species and sequences database accession numbers used in this study for genus *Diaporthe*. Ex-type isolates are given in **bold**. (Note: sequences marked with the symbol – were not used)

Species	Culture	Host	Location	ITS	*tef1-α*	*tub*	*cal*	*his*
Diaporthe absenteum	**LC3429**	*Camellia sinensis*	China	KP267897	-	-	-	-
D. acaciarum	**CBS 138862**	*Acacia tortilis*	Tanzania	KP004460	-	-	-	-
D. acaciigena	**CBS129521**	*Acacia retinodes*	Australia	KC343005	-	-	-	-
D. acericola	**MFLUCC 17-0956**	*Acer negundo*	Italy	KY964224	-	-	-	-
D. acerina	**CBS 137.27**	*Acer saccharum*	-	KC343006	-	-	-	-
D.actinidiae	LP-2	*Actinidia* sp.	China	KX457968	-	-	-	-
	KFRD-7	*Actinidia deliciosa*	China	KX346886	-	-	-	-
	KFRD-8	*Actinidia chinensis*	China	KX346887	-	-	-	-
D. acutispora	LC 6161	*Coffea* sp.	China	KX986764	-	-	-	-
D. alleghaniensis	**CBS 495.72**	*Betula alleghaniensis*	Canada	KC343007	KC343733	KC343975	KC343249	KC343491
D. alnea	**CBS 146.46**	*Alnus* sp.	-	KC343008	-	-	-	-
	CBS 159.47	*Alnus* sp.	-	KC343009	-	-	-	-
D. ambigua	**CBS 114015**	*Pyrus communis*	South Africa	KC343010	-	-	-	-
	CBS 117167	*Aspalathus linearis*	South Africa	KC343011	-	-	-	-
D. ampelina	**CBS 114016**	*Vitis vinifera*	USA	AF230751	-	-	-	-
	CBS 114867	*Vitis vinifera*	Turkey	KC343017	-	-	-	-
D. amygdali	**CBS 126679**	*Prunus dulcis*	Portugal	KC343022	-	-	-	-
	CBS 126680	*Prunus dulcis*	Portugal	KC343023	-	-	-	-
D. anacardii	**CBS 720.97**	*Anacardium occidentale*	East Africa	KC343024	KC343750	KC343992	KC343266	KC343508
D. angelicae	**CBS 111592**	*Heracleum sphondylium*	Austria	KC343027	-	-	-	-
	CBS 123215	*Foeniculum vulgare*	Portugal	KC343028	-	-	-	-
D. apiculatum	**LC 3418**	*Camellia sinensis*	China	KP267896	-	-	-	-
D. aquatica	**IFRDCC 3051**	Aquatic habitat	China	JQ797437	-	-	-	-
D. arctii	**CBS 139280**	*Arctium lappa*	Germany	KJ590736	-	-	-	-
	CBS 136.25	*Arctium* sp.	-	KC343031	-	-	-	-
D. arecae	CBS 161.64	*Areca catechu*	India	FJ889452	-	-	-	-
D. arengae	**CBS 114979**	*Arenga engleri*	China	KC343034	-	-	-	-
D. aseana	**MFLUCC-12–0299**	Uknown dead leaf	Thailand	KT459414	-	-	-	-
D. asheicola	**CBS 136967**	*Vaccinium ashei*	Chile	KJ160562	-	-	-	-
D. aspalathi	CBS 117168	*Aspalathus linearis*	South Africa	KC343035	-	-	-	-
	CBS 117169	*Aspalathus linearis*	South Africa	KC343036	-	-	-	-

Table 7 - Continued

D. australafricana	**CBS 111886**	*Vitis vinifera*	Australia	KC343038	KC343764	KC344006	KC343280	KC343522
	CBS 113487	*Vitis vinifera*	South Africa	KC343039	KC343765	KC344007	KC343281	KC343523
D. averrohae	SCHM 3605	*Averrhoa carambola*	China	AY618930	-	-	-	-
D. baccae	**CBS 136972**	*Vaccinium corymbosum*	Italy	KJ160565	-	-	-	-
D. batatas	**CBS 122.21**	*Ipomoea batatas*	USA	KC343040	-	-	-	-
D. beckhausii	CBS 138.27	*Viburnum* sp.	-	KC343041	KC343767	KC344009	KC343283	KC343525
D. beilharziae	BRIP 54792	*Indigofera australis*	Australia	JX862529	-	-	-	-
D. benedicti	**ATCC MYA-4970**	-	-	KM669929	-	-	-	-
D. betulae	**CFCC 50469**	*Betula platyphylla*	China	KT732950	-	-	-	-
D. betulicola	**CFCC 51128**	*Betula albosinensis*	China	KX024653	-	-	-	-
D. bicincta	**CBS 121004**	*Juglans sp.*	USA	KC343134	KC343860	KC344102	KC343376	KC343618
D. biconispora	ZJUD60	*Citrus sinensis*	China	KJ490595	-	-	-	-
	ZJUD62	*Citrus grandis*	China	KJ490597	-	-	-	-
D. biguttulata	**ZJUD47**	*Citrus limon*	China	KJ490582	-	-	-	-
	ZJUD48	*Citrus limon*	China	KJ490583	-	-	-	-
D. biguttusis	**CGMCC 3.17081**	*Lithocarpus glabra*	China	KF576282	-	-	-	-
	CGMCC 3.17082	*Lithocarpus glabra*	China	KF576283	-	-	-	-
D. brasiliensis	**CBS 133183**	*Aspidospema tomentosum*	Brazil	KC343042	-	-	-	-
	LGMF926	*Aspidospema tomentosum*	Brazil	KC343043	-	-	-	-
D. caatingaensis	**CBS 141542**	*Pilosocereus gounellei*	Brazil	KY085928	-	-	-	-
D. campothecae	**SCHM 3611**	*Campotheca acuminate*	China	AY622996	-	-	-	-
D. canthii	**CBS 132533**	*Canthium inerne*	South Africa	JX069864	-	-	-	-
D. carpini	CBS 114437	*Carpinus betulus*	Sweden	KC343044	-	-	-	-
D. cassines	**CBS 136440**	*Cassine peragua*	South Africa	KF777155	-	-	-	-
D. caulivora	**CBS 127268**	*Glycine max*	Croatia	KC343045	-	-	-	-
	CBS 178.55	*Glycine soja*	Canada	KC343046	-	-	-	-
D. celastrina	**CBS 139.27**	*Celastrus scandens*	-	KC343047	KC343773	KC344015	KC343289	KC343531
D. chamaeropis	CBS 454.81	*Chamaerops humilis*	Greece	KC343048	KC343774	KC344016	KC343290	KC343532
	CBS 753.70	*Spartium junceum*	Croatia	KC343049	KC343775	KC344017	KC343291	KC343533
D. charlesworthii	**BRIP 54884m**	*Rapistrum rugostrum*	Australia	KJ197288	-	-	-	-
D. chimonanthi	**SCHM 3614**	*Chimananthus praecox*	China	AY622993	-	-	-	-

Table 7 - Continued

D. cichorii	**MFLUCC 17-1023**	*Cichorium intybus*	Italy	KY964220	-	-	-	-
D. cissampeli	**CBS 141331**	*Cissampelos capensis*	South Africa	KX228273	-	-	-	-
D. cinerascens	CBS 719.96	*Ficus carica*	Bulgaria	KC343050	KC343776	KC344018	KC343292	KC343534
D. citri	**AR3405**	*Citrus* sp.	USA	KC843311	-	-	-	-
	LGMF946	*Citrus* sp.	Brazil	KC343053	-	-	-	-
D. citriasiana	**CBS 134240**	*Citrus unshiu*	China	JQ954645	-	-	-	-
D. citrichinensis	**CBS 134242**	*Citrus unshiu*	China	JQ954648	-	-	-	-
	ZJUD 34B	*Citrus unshiu*	China	KJ210539	-	-	-	-
D. conorum	MAFF 410330	*Abies firma*	Japan	AB201443	-	-	-	-
D. compacta	**LC3083**	*Camellia sinensis*	China	KP267854	-	-	-	-
D. convolvuli	CBS 124654	*Convolvulus arvensis*	Turkey	KC343054	-	-	-	-
D. crataegi	CBS 114435	*Crataegus oxyacantha*	Sweden	KC343055	-	-	-	-
D. crotalariae	**CBS 162.33**	*Crotalaria spectabilis*	USA	KC343056	-	-	-	-
D. cucurbitae	CBS 136.25	*Arctium* sp.	Unknown	KC343031	-	-	-	-
D. cuppatea	**CBS 117499**	*Aspalathus linearis*	South Africa	KC343057	-	-	-	-
D. cynaroidis	**CBS 122676**	*Protea cynaroides*	South Africa	KC343058	KC343784	KC344026	KC343300	KC343542
D. cytosporella	CBS 137020	*Citrus limon*	Spain	KC843307	-	-	-	-
	AR5149	*Citrus sinensis*	USA	KC843309	-	-	-	-
D. decedens	CBS 109772	*Corylus avellana*	Austria	KC343059	-	-	-	-
	CBS 114281	*Corylus avellana*	Sweden	KC343060	-	-	-	-
D. detrusa	CBS 109770	*Berberis vulgaris*	Austria	KC343061	-	-	-	-
	CBS 114652	*Berberis vulgaris*	Sweden	KC343062	-	-	-	-
D. diospyricola	**CPC 21169**	*Diospyros whyteana*	South Africa	KF777156	-	-	-	-
D. discoidispora	CGMCC 3.17254	*Citrus sinensis*	China	KJ490622	-	-	-	-
	LC3503	*Camellia sinensis*	China	KY011887	-	-	-	-
	ZJUD89	*Citrus unshiu*	China	KJ490624	-	-	-	-
D. dorycnii	**MFLUCC 17-1015**	*Dorycnium hirsutum*	Italy	KY964215	-	-	-	-
D. elaeagni	CBS 504.72	*Elaeagnus* sp.	Netherlands	KC343064	KC343790	KC344032	KC343306	KC343548
D. elaeagni-glabrae	**LC4802**	*Elaeagnus glabra*	China	KX986779	-	-	-	-
D. ellipicola	**CGMCC 3.17084**	*Lithocarpus glabra*	China	KF576270	-	-	-	-
	CGMCC 3.17085	*Lithocarpus glabra*	China	KF576271	-	-	-	-
D. endophytica	**CBS 133811**	*Schinus terebinthifolius*	Brazil	KC343065	-	-	-	-
	LGMF911	*Schinus terebinthifolius*	Brazil	KC343066	-	-	-	-
D. eres	**CBS 138594**	*Ulmus laevis*	Germany	KJ210529	KJ210550	KJ420799	KJ434999	KC343574
	CBS 439.82	*Cotoneaster* sp.	Scotland	KC343090	KC343816	KC344058	KC343332	KJ420850

Table 7 - Continued

	DNP128	*Castaneae mollissimae*	China	JF957786	KJ210561	KJ420801	KJ435040	KJ420852
D. eucalyptorum	**CBS 132525**	*Eucalyptus* sp.	Australia	JX069862	-	-	-	-
D. eucomiae	**SCHM 0020**	*Eucommia ulmoides*	China	AY601921	-	-	-	-
D. eucomiicola	SCHM 3607	*Eucommia ulmoides*	China	AY578071	-	-	-	-
D. eugeniae	CBS 444.82	*Eugenia aromatica*	Indonesia	KC343098	-	-	-	-
D. fibrosa	CBS 109751	*Rhamnus cathartica*	Austria	KC343099	-	-	-	-
	CBS 113830	*Rhamnus cathartica*	Sweden	KC343100	-	-	-	-
D. foeniculina	**CBS 123208**	*Foeniculum vulgare*	Portugal	KC343104	KC343830	KC344072	KC343346	KC343588
	CBS 123209	*Foeniculum vulgare*	Portugal	KC343105	KC343831	KC344073	KC343347	KC343589
	CBS 187.27	*Camellia sinensis*	Italy	KC343107	KC343833	KC344075	KC343349	KC343591
D. fraxini-angustifoliae	**BRIP 54781**	*Fraxinus angustifolia*	Australia	JX862528	-	-	-	-
D. fusicola	**CGMCC 3.17088**	*Lithocarpus glabra*	China	KF576263	-	-	-	-
	CGMCC 3.17087	*Lithocarpus glabra*	China	KF576281	-	-	-	-
D. ganjae	**CBS 180.91**	*Cannabis sativa*	USA	KC343112	KC343838	KC344080	KC343354	KC343596
D. garathjonesii	**MFLUCC 12-0542**[a]	Unknown dead leaf	Thailand	KT459423	-	-	-	-
D. gardeniae	CBS 288.56	*Gardenia florida*	Italy	KC343113	-	-	-	-
D.glabrae	**SCHM 3622**	*Bougainvillea glabra*	China	AY601918	-	-	-	-
D. goulteri	**BRIP 55657**[a]	*Helianthus annuus*	Australia	KJ197290	-	-	-	-
D. gulyae	BRIP 53158	*Helianthus annuus*	Australia	JF431284	-	-	-	-
	BRIP 54025	*Helianthus annuus*	Australia	JF431299	-	-	-	-
D. helianthi	CBS 344.94	*Helianthus annuus*	-	KC343114	-	-	-	-
	CBS 592.81	*Helianthus annuus*	Serbia	KC343115	-	-	-	-
D. helicis	**CBS 138596**	*Hedera helix*	France	KJ210538	-	-	-	-
D. hickoriae	**CBS 145.26**	*Carya glabra*	USA	KC343118	-	-	-	-
D. hongkongensis	**CBS 115448**	*Dichroa febrífuga*	Hong Kong	KC343119	-	-	-	-
D. hordei	CBS 481.92	*Hordeum vulgare*	Norway	KC343120	-	-	-	-
D. impulsa	CBS 114434	*Sorbus aucuparia*	Sweden	KC343121	-	-	-	-
	CBS 141.27	*Sorbus americana*	-	KC343122	-	-	-	-
D. incompleta	**LC6754**	*Camellia sinensis*	China	KX986794	-	-	-	-
D. inconspicua	**CBS 133813**	*Maytenus ilicifolia*	Brazil	KC343123	KC343849	KC344091	KC343365	KC343607
	LGMF922	*Spondias mombin*	Brazil	KC343124	KC343850	KC344092	KC343366	KC343608
D. infecunda	**CBS 133812**	*Schinus terebinthifolius*	Brazil	KC343126	KC343852	KC344094	KC343368	KC343610
	LGMF908	*Schinus terebinthifolius*	Brazil	KC343127	KC343858	KC344100	KC343374	KC343616
D. isoberliniae	**CPC 22549**	*Isoberlinia angolensis*	Zambia	KJ869133	-	-	-	-

Table 7 - Continued

D. juglandicola	**CFCC 51134**	*Juglans mandshurica*	China	KU985101	-	-	-	-
D. juglandina	CBS 121004	*Juglans sp.*	USA	KC343134	KC343860	KC344102	KC343376	KC343618
D. kochmanii	**BRIP 54033**	*Helianthus annuus*	Australia	JF431295	-	-	-	-
	BRIP 54034	*Helianthus annuus*	Australia	JF431296	-	-	-	-
D. kongii	**BRIP 54031**	*Helianthus annuus*	Australia	JF431301	-	-	-	-
	BRIP 54032	*Helianthus annuus*	Australia	JF431300	-	-	-	-
D. kyushuensis	**STE-U 2675**	-	-	AF230749	-	-	-	-
D. lagerstroemiae	**SCHM 3608**	*Lagerstroemia indica*	China	AY622994	-	-	-	-
D. leucospermi	**CBS 111980**	*Leucospermum sp.*	Australia	JN712460	KY435632	KY435673	KY435663	KY435653
D. liquidambaris	**SCHM 3621**	*Liquidambar formosana*	China	AY601919	-	-	-	-
D. litchicola	**BRIP 54900**	*Litchi chinensis*	Australia	JX862533	-	-	-	-
D. lithocarpus	**CGMCC3.15175**	*Lithocarpus glabra*	China	KC153104	-	-	-	-
	LC0785	*Lithocarpus glabra*	China	KF576274	-	-	-	-
D. longicicola	**CGMCC 3.17089**	*Lithocarpus glabra*	China	KF576267	-	-	-	-
	CGMCC 3.17090	*Lithocarpus glabra*	China	KF576268	-	-	-	-
D. longicolla	**ATCC 60325**	*Glyine max*	USA	KJ590728	-	-	-	-
D. longispora	**CBS 194.36**	*Ribes sp.*	Canada	KC343135	-	-	-	-
D. lonicerae	**MFLUCC 17-0963**	*Lonicera sp.*	Italy	KY964190	-	-	-	-
D. loropetali	**SCHM 3615**	*Loropetalum chinense*	China	AY601917	-	-	-	-
D. lusitanicae	**CBS 123212**	*Foeniculum vulgare*	Portugal	KC343136	-	-	-	-
	CBS 123213	*Foeniculum vulgare*	Portugal	KC343137	-	-	-	-
D. macintoshii	**BRIP 55064[a]**	*Rapistrum rugostrum*	Australia	KJ197289	-	-	-	-
D. magnoliicola	**SCHM 3001**	*Magnolia coco*	China	AY622995	-	-	-	-
D. mahothocarpus	**CGMCC 3.15181**	*Lithocarpus glabra*	China	KC153096	-	-	-	-
D. malorum	**CBS142383**	*Malus domestica*	Portugal	KY435638	KY435627	KY435668	KY435658	KY435648
D. maritima	**NB365-71I**	*Picea rubens*	Canada	KU552025	-	-	-	-
	NB464-3[a]	*Picea rubens*	Canada	KU552027	-	-	-	-
D. manihotia	CBS 505.76	*Manihot utilissima*	Rwanda	KC343138	KC343864	KC344106	KC343380	KC343622
D. masirevicii	BRIP 57330	*Chrysanthemoides monilifera*	Australia	KJ197275	-	-	-	-
	BRIP 57892[a]	*Helianthus annuus*	Australia	KJ197277	-	-	-	-
D. mayteni	**CBS 133185**	*Maytenus ilicifolia*	Brazil	KC343139	-	-	-	-
D. maytenicola	**CBS 136441**	*Maytenus acuminata*	South Africa	KF777157	-	-	-	-
D. megalospora	CBS 143.27	*Sambucus canadensis*	-	KC343140	-	-	-	-
D. melonis	CBS 435.87	*Glycine soja*	Indonesia	KC343141	-	-	-	-

Table 7 - Continued

	CBS 507.78	*Cucumis melo*	USA	KC343142	-	-	-	-
D. meridionalis	**227-9**	*Glycine max*	-	AJ312361	-	-	-	-
D. michelina	**SCHM 3603**	*Michelia alba*	Cina	AY620820	-	-	-	-
D. middletonii	**BRIP 54884e**	*Rapistrum rugostrum*	Australia	KJ197286	-	-	-	-
	BRIP 57329	*Chrysanthemoides monilifera* subsp. *rotundata*	Australia	KJ197285	-	-	-	-
D. multigutullata	**ZJUD98**	*Citrus grandis*	China	KJ490633	-	-	-	-
D. miriciae	BRIP 56918[a]	*Vigna radiata*	Australia	KJ197284	-	-	-	-
	BRIP 55662c	*Glycine max*	Asutralia	KJ197283	-	-	-	-
	BRIP 54736j	*Helianthus annuus*	Australia	KJ197282	-	-	-	-
D. momicola	**MFLUCC 16-0113**	*Prunus persica*	China	KU557563	-	-	-	-
D. musigena	**CBS 129519**	*Musa* sp.	Australia	KC343143	-	-	-	-
D. neilliae	CBS 144.27	*Spiraea* sp.	-	KC343144	-	-	-	-
D. neoarctii	**CBS 109490**	*Ambrosia trifida*	USA	KC343145	-	-	-	-
D. neoraonikayaporum	**MFLUCC 14–1136**	*Tectona grandis*	Thailand	KU712449	-	-	-	-
D. nobilis	CBS 116953	*Pyrus pyrifolia*	New Zealand	KC343147	-	-	-	-
	CBS 124030	*Malus pumila*	New Zealand	KC343149	-	-	-	-
D. nomurai	CBS 157.29	*Morus* sp.	Japan	KC343154	-	-	-	-
D. nothofagi	**BRIP 54801**	*Nothofagus cunninghamii*	Australia	JX862530	-	-	-	-
D. novem	**CBS 127270**	*Glycine max*	Croatia	KC343156	-	-	-	-
	CBS 127271	*Glycine max*	Croatia	KC343157	-	-	-	-
D. ocoteae	**CBS 141330**	*Ocotea obtusata*	France	KX228293	-	-	-	-
D. oncostoma	CBS 100454	*Robinia pseudoacacia*	Germany	KC343160	-	-	-	-
	CBS 809.85	*Ilex aquifolium*	Germany	KC343163	-	-	-	-
D. oraccini	**CGMCC 3.17531**	*Camellia sinensis*	China	KP267863	-	-	-	-
D. ovalispora	**ZJUD93**	*Citrus limon*	China	KJ490628	-	-	-	-
D. ovoicicola	**CGMCC 3.17092**	*Lithocarpus glabra*	China	KF576264	-	-	-	-
	CGMCC 3.17093	*Lithocarpus glabra*	China	KF576265	-	-	-	-
D. oxe	CBS 133187	*Maytenus ilicifolia*	Brazil	KC343165	-	-	-	-
	CBS 133186	*Maytenus ilicifolia*	Brazil	KC343164	-	-	-	-
D. padi var. padi	CBS 114649	*Alnus glutinosa*	Sweden	KC343170	-	-	-	-
	CBS 114200	*Prunus padus*	Sweden	KC343169	-	-	-	-

Table 7 - Continued

D. paranensis	**CBS 133184**	*Maytenus ilicifolia*	Brazil	KC343171	-	-	-	-
D. parapterocarpi	**CPC 22729**	*Pterocarpus brenanii*	Zambia	KJ869138	-	-	-	-
D. pascoei	**BRIP 54847**	*Persea americana*	Australia	JX862532	-	-	-	-
D. passiflorae	**CBS 132527**	*Passiflora edulis*	South America	JX069860	-	-	-	-
D. passifloricola	**CBS 141329**	*Passiflora foetida*	Malaysia	KX228292	-	-	-	-
D. penetriteum	LC3215	*Camellia sinensis*	China	KP267879	-	-	-	-
	LC3394	*Camellia sinensis*	China	KP267893	-	-	-	-
D. perjuncta	**CBS 109745**	*Ulmus glabra*	Austria	KC343172	-	-	-	-
D. perseae	CBS 151.73	*Persea gratissima*	Netherlands	KC343173	-	-	-	-
D. pescicola	MFLUCC 16-0105	*Prunus persica*	China	KU557555	-	-	-	-
D. phaseolorum	**CBS 139281**	*Phaseolus vulgaris*	USA	KJ590738	-	-	-	-
	CBS 116019	*Caperonia palustris*	USA	KC343175	-	-	-	-
D. phragmitis	**CBS 138897**	*Phragmites australis*	China	KP004445	-	-	-	-
D. phyllanthicola	**SCHM 3680**	*Phyllanthus emblica*	China	AY620819	-	-	-	-
D. podocarpi-macrophylli	**LC6155**	*Podocarpus macrophyllus*	Japan	KX986774	-	-	-	-
D. pseudomangiferae	**CBS 101339**	*Mangifera indica*	Dominican Republic	KC343181	-	-	-	-
	CBS 388.89	*Mangifera indica*	Mexico	KC343182	-	-	-	-
D. pseudophoenicicola	CBS 176.77	*Mangifera indica*	Iraq	KC343183	-	-	-	-
	CBS 462.69	*Phoenix dactylifera*	Spain	KC343184	-	-	-	-
D. pseudotsugae	**MFLU 15-3228**	*Pseudotsuga menziesii*	Italy	KY964225	-	-	-	-
D. psoraleae	**CPC 21634**	*Psoralea pinnata*	South Africa	KF777158	-	-	-	-
D. psoraleae-pinnatae	**CPC 21638**	*Psoralea pinnata*	South Africa	KF777159	-	-	-	-
D. pterocarpi	**MFLUCC 10-0571**	*Pterocarpus indicus*	Thailand	JQ619899	-	-	-	-
	MFLUCC 10-0575	*Pterocarpus indicus*	Thailand	JQ619901	-	-	-	-
D. pterocarpicola	**MFLUCC 10-0580**[b]	*Pterocarpus indicus*	Thailand	JQ619888	-	-	-	-
	CBS 135432	*Pterocarpus indicus*	Thailand	JQ619887	-	-	-	-
D. pulla	**CBS 338.89**	*Hedera helix*	Yugoslavia	KC343152	-	-	-	-
D. pustulata	CBS 109742	*Acer pseudoplatanus*	Austria	KC343185	KC343911	KC344153	KC343427	KC343669
	CBS 109784	*Prunus padus*	Austria	KC343187	KC343913	KC344155	KC343429	KC343671
D. pyracanthae	**CBS142384**	*Pyracantha coccinea*	Portugal	KY435635	KY435625	KY435666	KY435656	KY435645
D. ravenica	**MFLUCC 15–0479**	*Tamarix* sp.	Italy	KU900335	-	-	-	-
D. rhoina	CBS 146.27	*Rhus toxicodendron*	-	KC343189	-	-	-	-

Table 7 - Continued

D. rhusicola	CBS 129528	*Rhus pendulina*	South Africa	JF951146	-	-	-	-
D. rostrata	**CFCC 50062**	*Juglans mandshurica*	China	KP208847	-	-	-	-
	CFCC 50063	*Juglans mandshurica*	China	KP208848	-	-	-	-
D. rudis	CBS 100170	*Fraxinus excelsior*	Netherlands	KC343230	KC343956	KC344198	KC343472	KC343714
	CBS 113201	*Vitis vinifera*	Portugal	KC343234	KC343960	KC344202	KC343476	KC343718
	CBS 266.85	*Lupinus angustifolius*	Australia	KC343237	KC343963	KC344205	KC343479	KC343721
D. saccarata	**CBS 116311**	*Protea repens*	South Africa	KC343190	-	-	-	-
D. sackstonii	**BRIP 54669[b]**	*Helianthus annuus*	Australia	KJ197287	-	-	-	-
D. salicicola	**BRIP 54825**	*Salix purpurea*	Australia	JX862531	-	-	-	-
D. schini	**CBS 133181**	*Schinus terebinthifolius*	Brazil	KC343191	-	-	-	-
	LGMF910	*Schinus terebinthifolius*	Brazil	KC343192	-	-	-	-
D. schoeni	**MFLU 15-1279**	*Schoenus nigricans*	Italy	KY964226	-	-	-	-
D. sclerotioides	**CBS 296.67**	*Cucumis sativus*	Netherlands	KC343193	-	-	-	-
	CBS 710.76	*Cucumis sativus*	Netherlands	KC343194	-	-	-	-
D. scobina	CBS 251.38	*Fraxinus excelsior*	Scotland	KC343195	-	-	-	-
D. sennae	**CFCC 51636**	*Senna bicapsularis*	China	KY203724	-	-	-	-
D. sennicola	**CFCC 51634**	*S. bicapsularis*	China	KY203722	-	-	-	-
D. serafiniae	BRIP 54136	*Lupinus albus 'Rosetta'*	Australia	KJ197273	-	-	-	-
	BRIP 55665[a]	*Helianthus annuus*	Australia	KJ197274	-	-	-	-
D.siamensis	**MFLUCC 10-0573[a]**	*Dasymaschalon* sp.	Thailand	JQ619879	-	-	-	-
	MFLUCC 10-0573[b]	*Dasymaschalon* sp.	Thailand	JQ619880	-	-	-	-
	MFLUCC 10-0573[c]	*Dasymaschalon* sp.	Thailand	JQ619881	-	-	-	-
D. sojae	**CBS 139282**	*Glycine max*	USA	KJ590719	-	-	-	-
	MAFF 410444	*Cucumis melo*	Japan	KJ590714	-	-	-	-
D. spartinicola	CBS 140003	*Spartium junceum*	Spain	KR611879	-	-	-	-
D. sterilis	CBS 136969	*Vaccinium corymbosum*	Italy	KJ160579	-	-	-	-
D. stewartii	**CBS 193.36**	*Cosmos bipinnatus*	USA	FJ889448	-	-	-	-
D. stictica	CBS 370.54	*Buxus sempervirens*	Italy	KC343212	KC343938	KC344180	KC343454	KC343696
D. subclavata	ZJUD83	*Citrus grandis*	China	KJ490618	-	-	-	-
	ZJUD95	*Citrus unshiu*	China	KJ490630	-	-	-	-
D. subordinaria	CBS 101711	*Plantago lanceolata*	New Zealand	KC343213	-	-	-	-
	CBS 464.90	*Plantago lanceolata*	South Africa	KC343214	-	-	-	-
D. taoicola	MFLUCC 16-0117	*Prunus persica*	China	KU557567	-	-	-	-
D. tecomae	CBS 100547	*Tabebuia* sp.	Brazil	KC343215	KC343941	KC344183	KC343457	KC343699

Table 7 - Continued

D. tectonae	**MFLUCC 12–0777**	*Tectona grandis*	Thailand	KU712430	-	-	-	-
D. tectonendophytica	**MFLUCC 13–0471**	*Tectona grandis*	Thailand	KU712439	-	-	-	-
D. tecctonigena	**MFLUCC 12–0767**	*Tectona grandis*	Thailand	KU712429	-	-	-	-
D. terebinthifolii	**CBS 133180**	*Schinus terebinthifolius*	Brazil	KC343216	-	-	-	-
	LGMF907	*Schinus terebinthifolius*	Brazil	KC343217	-	-	-	-
D. terntroemia	**CGMCC 3.15183**	*Ternstroemia gymnanthera*	China	KC153098	-	-	-	-
	CGMCC 3.15184	*Ternstroemia gymnanthera*	China	KC153099	-	-	-	-
D. thunbergii	**MFLUCC10-576[a]**	*Thunbergia laurifolia*	Thailand	JQ619893	-	-	-	-
	MFLUCC10-576[b]	*Thunbergia laurifolia*	Thailand	JQ619894	-	-	-	-
	MFLUCC10-576[c]	*Thunbergia laurifolia*	Thailand	JQ619895	-	-	-	-
D. thunbergiicola	**MFLU 12-0033**	*Thunbergia laurifolia*	Thailand	KP715097	-	-	-	-
D. torilicola	**MFLUCC 17-1051**	*Torilis arvensis*	Italy	KY964212	-	-	-	-
D. toxica	**CBS 534.93**	*Lupinus angustifolius*	Australia	KC343220	KC343946	KC344188	KC343462	KC343704
D. tulliensis	**BRIP 62248[a]**	*Theobroma cacao*	Australia	KR936130	-	-	-	-
	LP-1	*Actinidia* sp.	China	KX457967	-	-	-	-
D. ueckerae	**CBS 139283**	*Cucumis melo*	USA	KJ590726	-	-	-	-
	LGMF947	*Glycine max*	Brazil	KC343203	-	-	-	-
D. unulata	**LC6624**	unkown	China	KX986798	-	-	-	-
D. unshiuensis	ZJUD49	*Fortunella margarita*	China	KJ490584	-	-	-	-
	ZJUD52	*Citrus unshiu*	China	KJ490587	-	-	-	-
D. vaccinii	CBS 122112	*Vaccinium macrocarpon*	USA	KC343224	KC343950	KC344192	KC343466	KC343708
	CBS 160.32	*Oxycoccus macrocarpos*	USA	KC343228	KC343954	KC344196	KC343470	KC343712
D. vangueriae	**CPC 22703**	*Vangueria infausta*	Zambia	KJ869137	-	-	-	-
D. vawdreyi	**BRIP 57887[a]**	*Psidium guajava*	Australia	KR936126	-	-	-	-
D. velutina	**LC441**	*Neolitsea sp.*	China	KX986790	-	-	-	-
D. vexans	CBS 127.14	*Solanum melongena*	USA	KC343229	-	-	-	-
	FAU597	*Solanum* sp.	Dominican Republic	KJ590734	-	-	-	-
D. virgiliae	**CMW 40748**	-	-	KP247566	-	-	-	-
	CMW 40755	-	-	KP247573	-	-	-	-

Table 7 - Continued

D. woodii	CBS 558.93	*Lupinus* sp.	Australia	KC343244	-	-	-	-
D. woolworthii	CBS 148.27	*Ulmus americana*	-	KC343245	-	-	-	-
D. xishuangbanica	**CGMCC 3.18282**	*Camellia sinensis*	China	KX986783	-	-	-	-
D. yunnanensis	**CGMCC 3.18289**	*Coffea* sp.	China	KX986796	-	-	-	-
Diaporthe cf. heveae 1	CBS 852.97	*Hevea brasiliensis*	Brazil	KC343116	-	-	-	-
Diaporthe cf. heveae 2	CBS 681.84	*Heveae brasiliensis*	India	KC343117	-	-	-	-
Diaporthe G-type	930811–14	*Prunus persica*	Japan	AB302255	-	-	-	-
Diaporthe G-type	930811–17	*Prunus persica*	Japan	GQ250201	-	-	-	-
Diaporthe W-type	P-Pt-16	*Prunus persica*	Japan	AB302245	-	-	-	-
Diaporthe W-type	P-Pt-19	*Prunus persica*	Japan	GQ250202	-	-	-	-
Diaporthe sp1	CBS 119639	*Homo sapiens*	Germany	KC343202	-	-	-	-
Diaporthe sp2	LGMF 932	*Maytenus ilicifolia*	Brazil	KC343204	-	-	-	-
Diaporthe sp3	CBS 287.29	*Pseudotsuga menziesii*	Scotland	KC343205	-	-	-	-
Diaporthe sp4	LGMF944	*Maytenus ilicifolia*	Brazil	KC343206	-	-	-	-
Diaporthe sp5	CBS 125575	*Acer opalus*	Italy	KC343207	-	-	-	-
Diaporthe sp6	G65-65	*Piper hispidum*	Brazil	JF767007	-	-	-	-
Diaporthe sp7	CBS 115584	*Maesa perlarius*	Hong Kong	KC343208	-	-	-	-
Diaporthe sp8	CBS 115595	*Maesa perlarius*	Hong Kong	KC343209	-	-	-	-
Diaporthe sp9	CBS 458.78	*Anacardium occidentale*	India	KC343210	-	-	-	-
Diaporthe sp10	LGMF 925	*Aspidosperma tomentosum*	Brazil	KC343211	-	-	-	-
Diaporthe sp11	ZJUD86	*Fortunella margarita*	China	KJ490621	-	-	-	-
Diaporthe sp12	FAU501	*Cucumis melo*	Trinidad	KJ590722	-	-	-	-
Diaporthe sp13	G27-60	*Piper hispidum*	Brazil	JF766998	-	-	-	-
Diaporthe sp14	G29-79	*Piper hispidum*	Brazil	JF767000	-	-	-	-
Diaporthe sp15	1308-05	*Aucuba japonica*	Japan	LC009377	-	-	-	-
Diaporthe sp16	MFLUCC 10-0582	*Aeschynanthus radicans*	Thailand	JQ619885	-	-	-	-
Diaporthe sp17	MFLUCC 10-0570	Dead wood-unknown	Thailand	JQ619877	-	-	-	-
Diaporthe sp18	CMT 71	*Phaseolus vulgaris*	Brazil	KP182396	-	-	-	-
Diaporthe sp19	CMT 41	*Phaseolus vulgaris*	Brazil	KP182391	-	-	-	-
Diaporthe sp20	**LC0771**	*Alnus* sp.	Italy	KX986799	-	-	-	-

Table 7 - Continued

Diaporthe sp21	**LC 6496**	*Camellia sinensis*	Italy	KX986781	-	-	-	-
Diaporthe sp22	**LC 6232**	*Theobroma cacao*	Italy	KX986797	-	-	-	-
Diaporthe sp23	**LC 8108**	*Theobroma cacao*	Italy	KY491543	-	-	-	-
Diaporthe sp24	**LC 8109**	*Theobroma cacao*	Italy	KY491544	-	-	-	-
Diaporthe sp25	**LC 8114**	unkown	Italy	KY491549	-	-	-	-
Diaporthe sp26	**LC 8115**	unkown	Italy	KY491550	-	-	-	-
Diaporthe sp27	**LGMF 947**	*Glycine max*	China	KC343203	-	-	-	-
Phomopsis amaranthicola	**ATCC 74226**	*Amaranthus* sp.	USA	AF079776	-	-	-	-
P. asparagi	MAFF 237556	*Actinidia deliciosa*	Japan	AB107885	-	-	-	-
	MAFF 237559	*Asparagus officinalis*	Japan	AB107886	-	-	-	-
P. azadirachtae	TN 01	*Azadirachta indica*	India	KC631323	-	-	-	-
P. bougainvilleicola	SCHM 3006	*Bougainvillea glabra*	China	AY601920	-	-	-	-
P. columnaris	**BPI 841341**	*Vaccinium vitis-idaea*	USA	AF439625	-	-	-	-
	TS 5	*Picea abies*	Lithuania	DQ093770	-	-	-	-
P. dauci	**CBS 315.49**	*Daucus carota*	Netherlands	FJ889451	-	-	-	-
P. emicis	BRIP 45089[a]	*Emex australis*	-	JF957784	-	-	-	-
	BRIP 45089[b]	-	-	JQ619898	-	-	-	-
P. mauritina	**Z031203**	*Zizyphus mauritiana*	China	EU012334	-	-	-	-
P. oryzae	IMI158929	-	-	AF079777	-	-	-	-
P. saccharata	(holotype)	*Protea repens*	South Africa	AF387817	-	-	-	-
Phomopsis sp1	Ph-AC002	*Acanthus* sp.	Portugal	GQ250216	-	-	-	-
Phomopsis sp2	Ph-AC003	*Acanthus* sp.	Portugal	GQ250217	-	-	-	-
Phomopsis sp3	Ph-C192/1	*Hydrangea macrophylla*	Portugal	GQ250224	-	-	-	-
Phomopsis sp4	Ph-C169/1	*Hydrangea macrophylla*	Portugal	GQ250218	-	-	-	-
Phomopsis sp5	Ph-C173/1	*Hydrangea macrophylla*	Portugal	GQ250219	-	-	-	-
Phomopsis sp6	Ph-C174/1	*Hydrangea macrophylla*	Portugal	GQ250220	-	-	-	-
Phomopsis sp7	Ph-C180/1	*Acer negundo*	Portugal	GQ250221	-	-	-	-
Phomopsis sp8	Ph-C188/1	*Hydrangea macrophylla*	Portugal	GQ250222	-	-	-	-
Phomopsis sp9	Ph-C189/1	*H. macrophylla*	Portugal	GQ250223	-	-	-	-

Table 7 - Continued

Phomopsis sp10	Ph-C194/1	*H. macrophylla*	Portugal	GQ250225	-	-	-	-
Phomopsis sp11	JMS2010i_151	*Vitis vinifera*	Portugal	GQ250226	-	-	-	-
Phomopsis sp12	JMS2010i_152	*Vitis vinifera*	Portugal	GQ250227	-	-	-	-
Phomopsis sp13	439B4	*Vitis vinifera*	Portugal	GQ250228	-	-	-	-
Phomopsis sp14	UCD181-Oe	*Olea europaea*	USA	JX515731	-	-	-	-
Phomopsis sp15	UCD182-Oe	*Olea europaea*	USA	JX515732	-	-	-	-
Phomopsis sp16	UCD213-Oe	*Olea europaea*	USA	JX515733	-	-	-	-
Phomopsis sp17	UCD233-Oe	*Olea europaea*	USA	JX515734	-	-	-	-
Phomopsis sp18	UCD237-Oe	*Olea europaea*	USA	JX515735	-	-	-	-
Phomopsis sp19	UCD248-Oe	*Olea europaea*	USA	JX515736	-	-	-	-
Phomopsis sp20	UCD278-Oe	*Olea europaea*	USA	JX515737	-	-	-	-
Phomopsis sp21	UCD580-Oe	*Olea europaea*	USA	JX515738	-	-	-	-
Phomopsis sp22	UCD1685SI	*Vitis vinifera*	USA	JX515738	-	-	-	-
Phomopsis sp23	CAL-5	*Vitis vinifera*	USA	FJ794470	-	-	-	-
Phomopsis sp24	CBS 117165	*Aspalathus linearis*	USA	AY745085	-	-	-	-

5.3.1. *Botryosphaeriaceae* phylogenies

Kimura 2-parameter model was used to infer the ITS and the ITS+*tef1-α* ML tree for *Botryosphaeria* (Kimura, 1980). For the ITS ML analysis, all *Botryosphaeria* isolates (M89, M126, M171, M210, M304, CAA767, CAA773, CAA784, CAA785 and CAA788) were clustered with the representative *Botryosphaeria dothidea* type with 51 % bootstrap support (Figure 1).

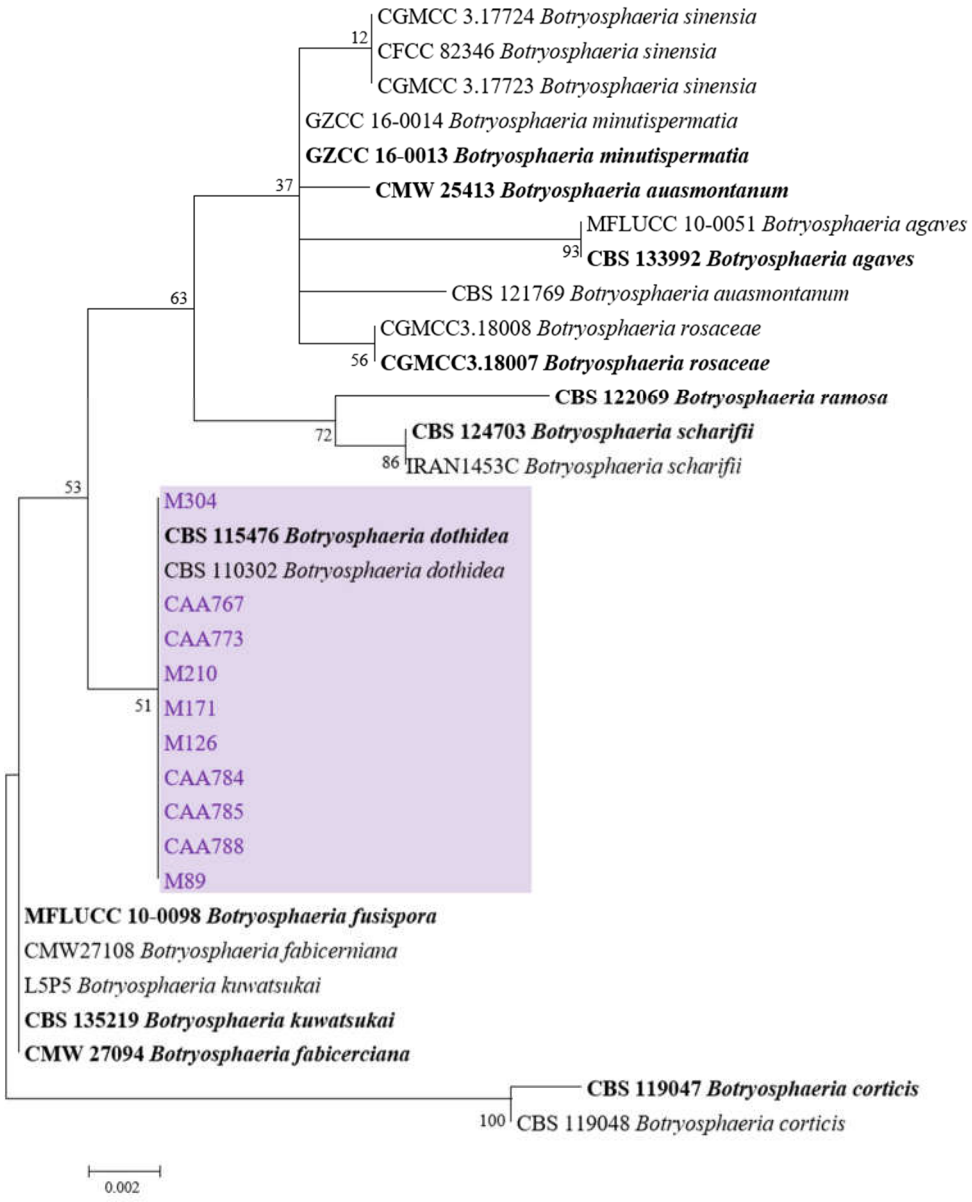

Figure 1 – ML phylogenetic tree of ITS region from *Botryosphaeria* species. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. Bootstrap values are shown next to the branches. Ex-type isolates are given **in bold.**

The multi-loci ML tree of ITS and *tef1-α* was in concordance with the ITS ML tree. All representative isolates were clustered with *Botryosphaeria dothidea* with a bootstrap support of 85 % (Figure 2)

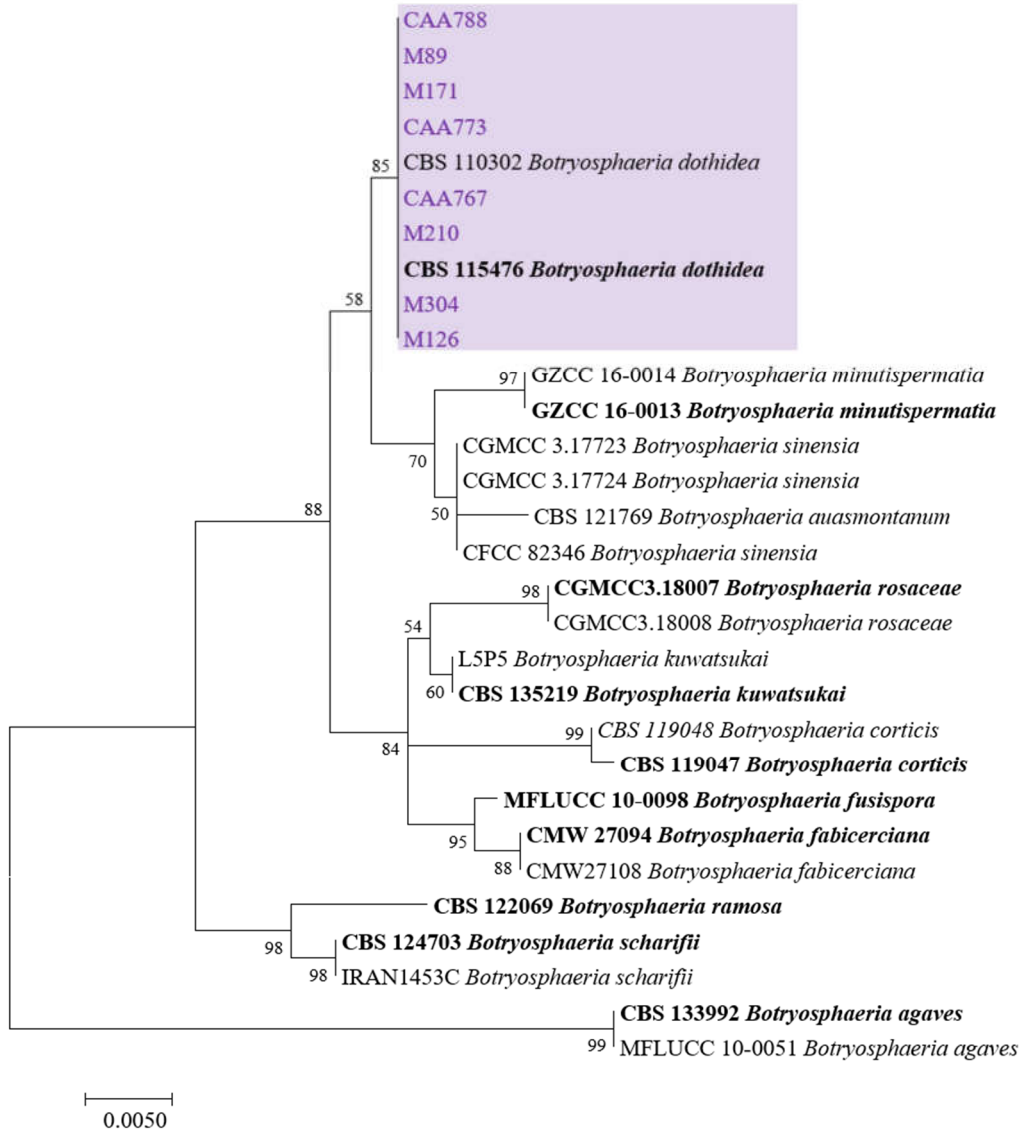

Figure 2 – ML phylogenetic tree obtained from combined analysis of ITS and *tef1-α* sequence data from species of *Botryosphaeria* based on the Kimura 2-parameter model. Bootstrap values are shown next to the branches. Ex-type isolates are given **in bold.**

The ITS ML tree of *Neofusicoccum* was constructed based on the Kimura 2-parameter model assuming a gamma distribution and invariant sites as determined by MEGA7. Tamura 3-parameter model assuming a gamma distribution was used to construct the ML tree combining ITS and *tef1-α* for *Neofusicoccum* (Tamura, 1992).

From the 22 *Neofusicoccum* isolates, 13 of them were identified as *N. parvum*. M23, M253, M22, M317, M189, M233, M165 and M240 clustered with the representative *N. parvum* type (CMW9081) with 47 % bootstrap support (Figure 3). M237, M328, M229, M97 and M336 clustered in a sub-clade with *Neofusicoccum parvum* (CBS 110301) with 65 % bootstrap support.

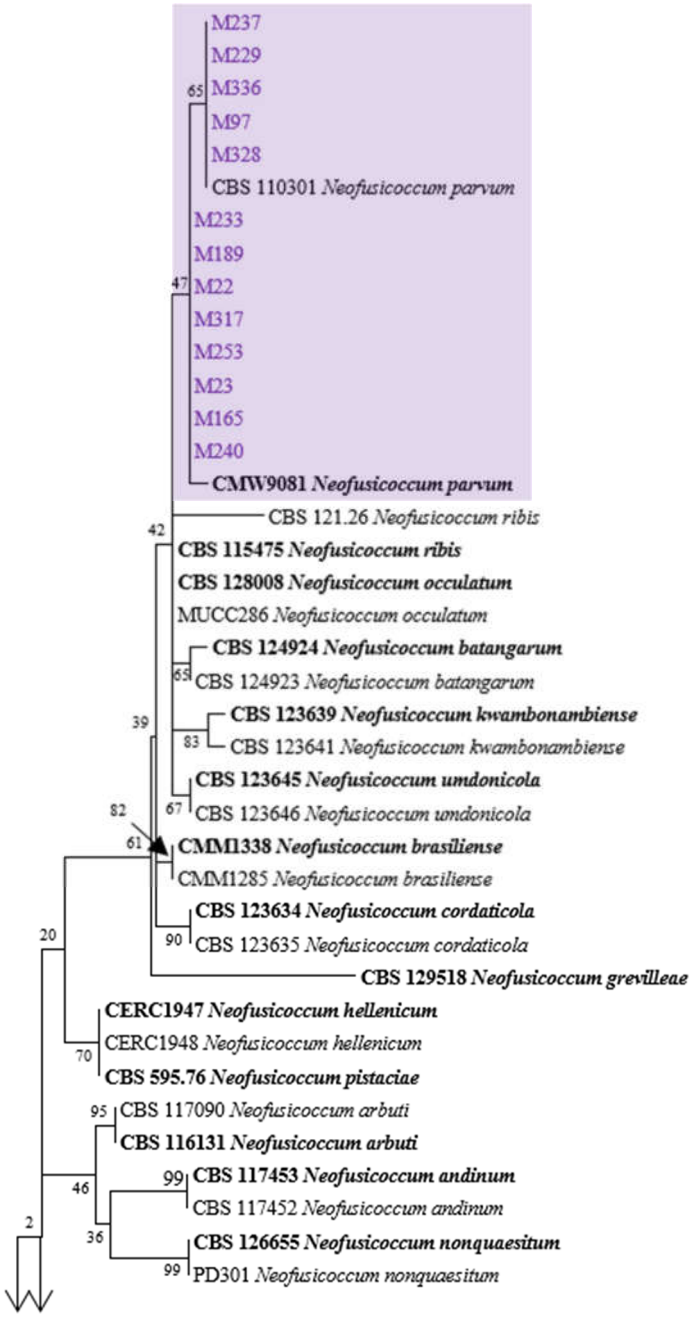

Figure 3 – ML phylogenetic tree of ITS region from *Neofusicoccum* species. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. Bootstrap values are shown next to the branches. Ex-type isolates are given **in bold.**

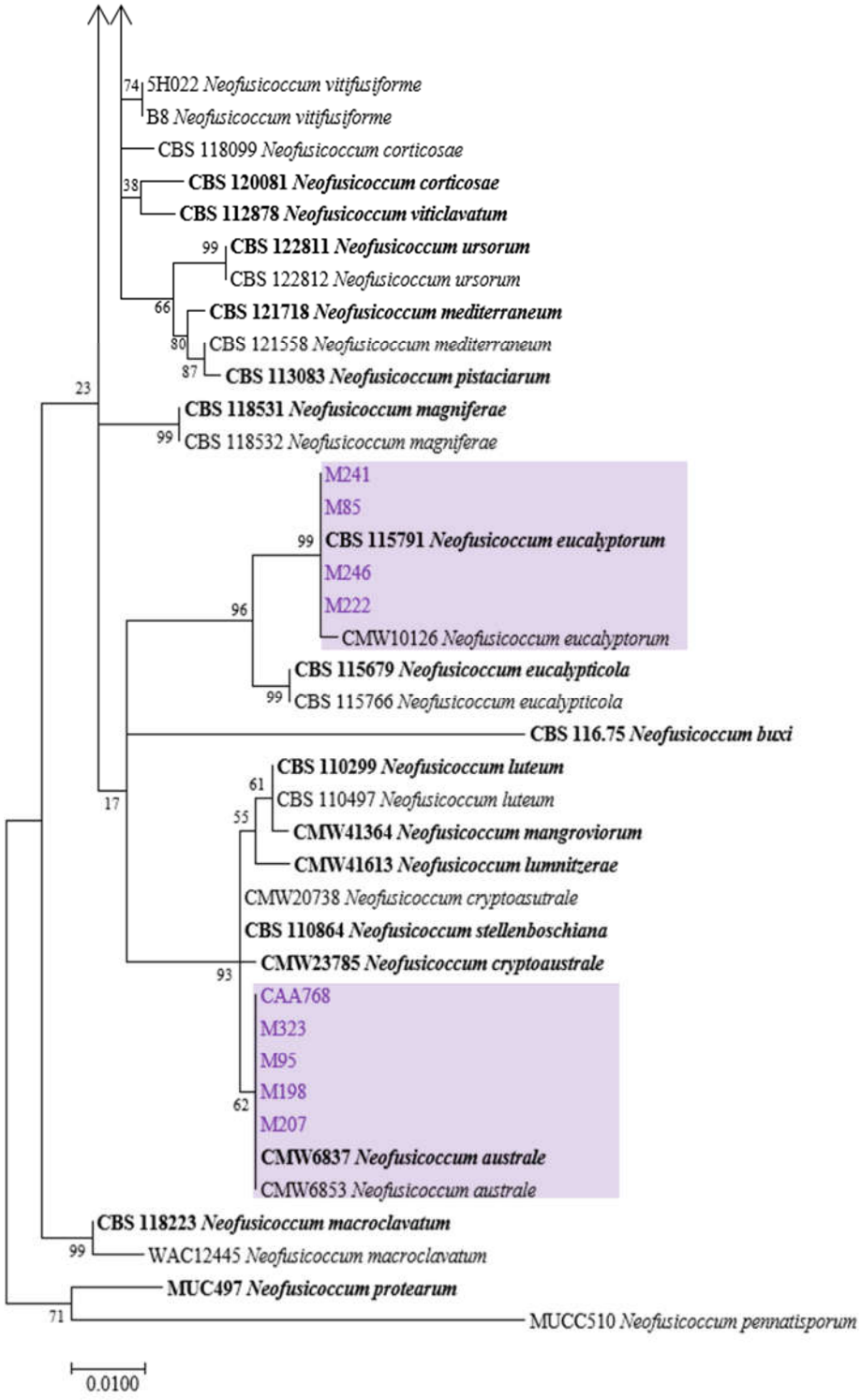

Figure 3 - continued

The *Neofusicoccum eucalyptorum* isolates obtained in this study (M222, M85, M241 and M246) were clustered with the representative type of *N.*

eucalyptorum (CBS 115791) with a 99 % bootstrap support. The *N. australe* clade grouped our isolates M323, M95, M198, M207 and CAA768 with 62 % of bootstrap support.

For an accurate and precise identification, 10 representative isolates were chosen for the construction of the multi-loci ML tree with ITS and *tef1-α* (Figure 4).

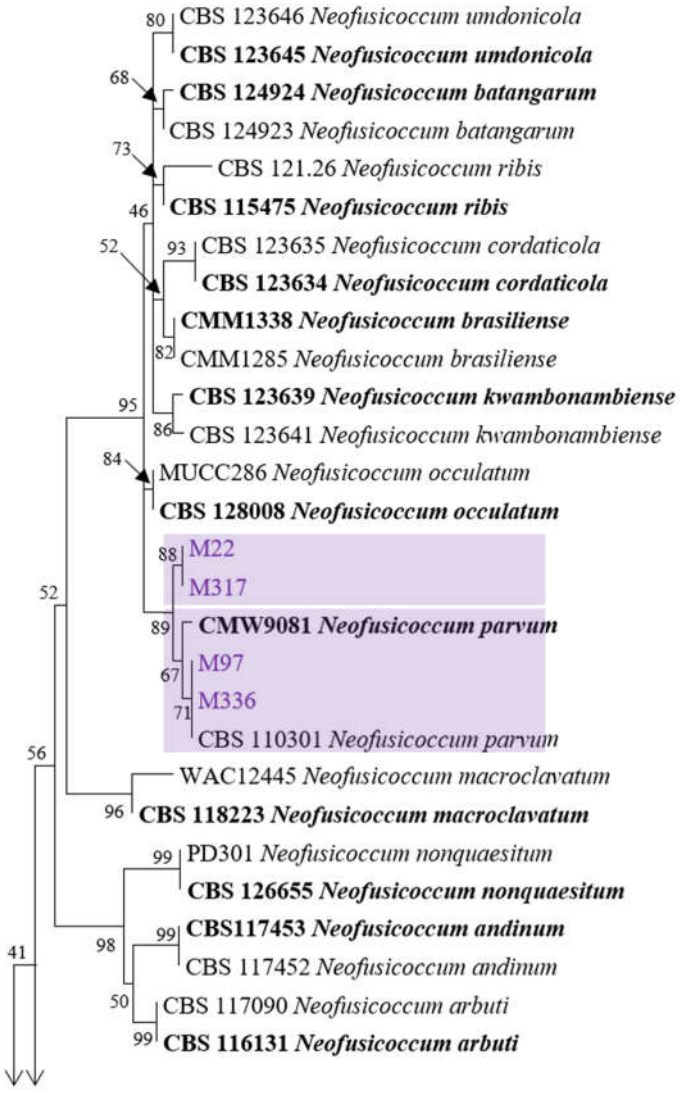

Figure 4 – ML phylogenetic tree obtained from combined analysis of ITS and *tef1-α* sequence data from species of *Neofusicoccum* based on the Tamura 3-parameter model. Bootstrap values are shown next to the branches. Ex-type isolates are given **in bold.**

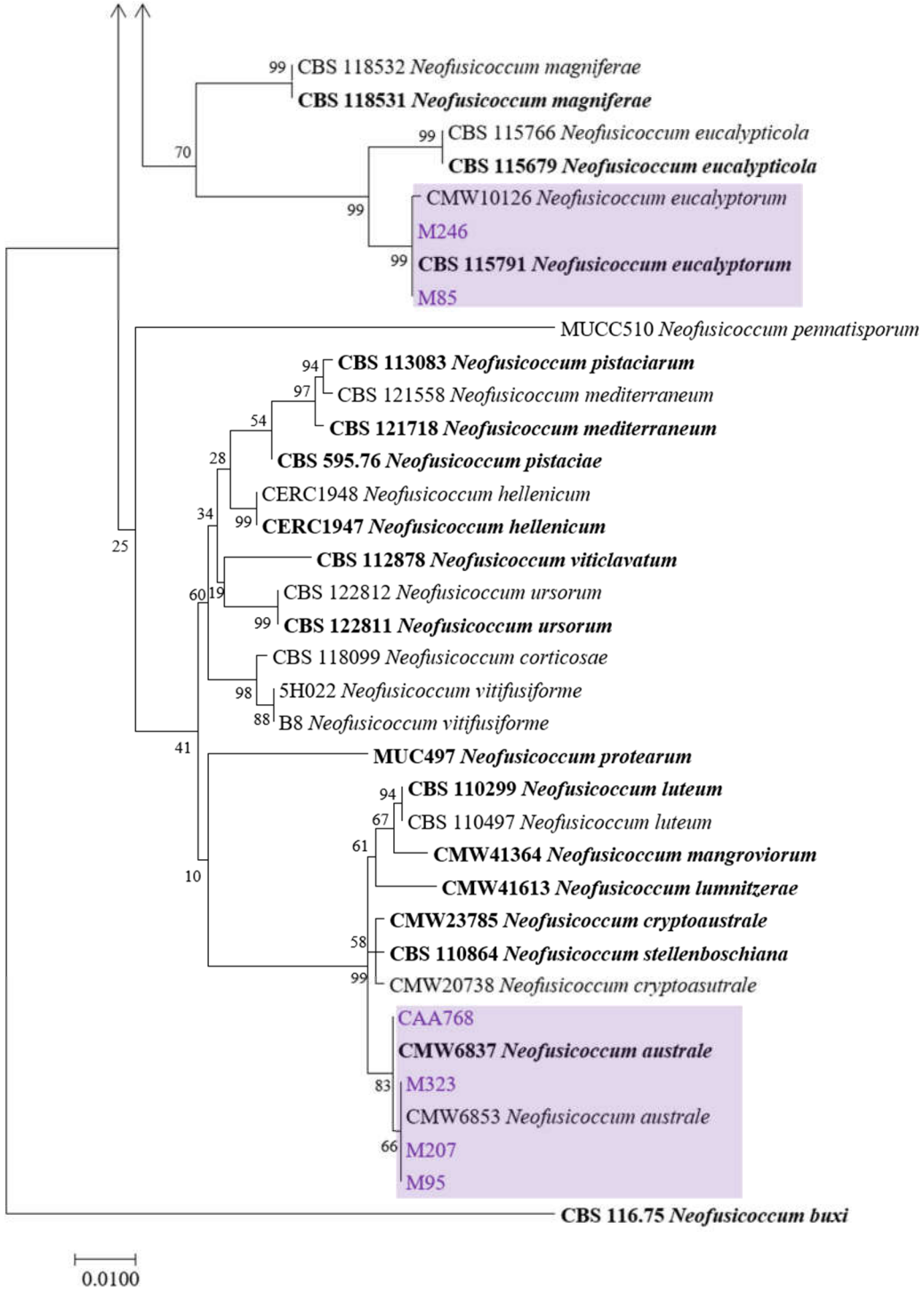

Figure 4 - continued

The results from the ITS ML analysis and the ITS combined with *tef1-α* are in concordance since isolates cluster in the same clades, although represented by different bootstrap values. According to the multi-loci analysis, isolates M22 and

M317 are phylogenetically closely related with *Neofusicoccum parvum*, as they come from the same branch with a bootstrap support of 89 %, but they seem slightly distinct once they cluster in a sub-clade. To better understand this separation, the differences in the nucleotide positions of both loci were compared (Table 8).

Table 8 - Nucleotide differences between *Neofucicoccum parvum* and M22, M317, M97, M336. Shared polymorphisms are highlighted in grey.

Locus		Isolates					
		N. parvum **CMW9081 ex-type**	*N. parvum* CBS 110301	M22	M317	M97	M336
ITS (514 bp)	49	A	T	A	A	A	A
	108	G	A	G	G	A	A
tef1-α (254 bp)	229	A	A	G	G	A	A
	233	A	A	C	C	A	A

A comparison of both loci sequences of *Neofusicoccum parvum* M22 and M317 with two *N. parvum* used in the multi-loci ML tree, showed that there are two unique polymorphisms in the sequences of *tef1-α* locus from M22 and M317, which may induce an intraspecific variability between the isolates.

5.3.2. *Diaporthe* phylogenies

The *Diaporthe* ITS ML analysis was based on the General Time Reversible model, assuming a gamma distribution and invariant sites (Nei & Kumar, 2000). The Tamura-Nei parameter model was used for the concatenated analysis of 5 loci (Tamura & Nei, 1993).

Results of the ITS phylogenetic analysis for the genus *Diaporthe* are shown in Figure 5. A clade containing the isolate *Phomopsis* sp4 (PhC169) with a bootstrap support of 33 %, grouped with 8 of our non-identified *Diaporthe* sp. 2 isolates (M162, M43, M156, M155, M291, M295, M298 and M164) (Figure 5). M101 and M134 are placed in the clade containing *Diaporthe eres* ex-types (CBS 138594 and CBS 439.82) with 83 % of bootstrap support. The clade has also grouped *D. lonicerae* and *D. asheicola*. The representative sequence of *Diaporthe vaccinii* (isolate type CBS 160.32) was grouped separately from all the isolates found in this study, hence no isolates were phylogenetically related with this

species. The *Diaporthe rudis* clade is supported by a bootstrap value of 81 % and grouped 5 of our isolates (M65, M15, CAA777, CAA789 and CAA790). The clade also grouped *D. cynaroidis* and *D. salicicola*, closely related species.

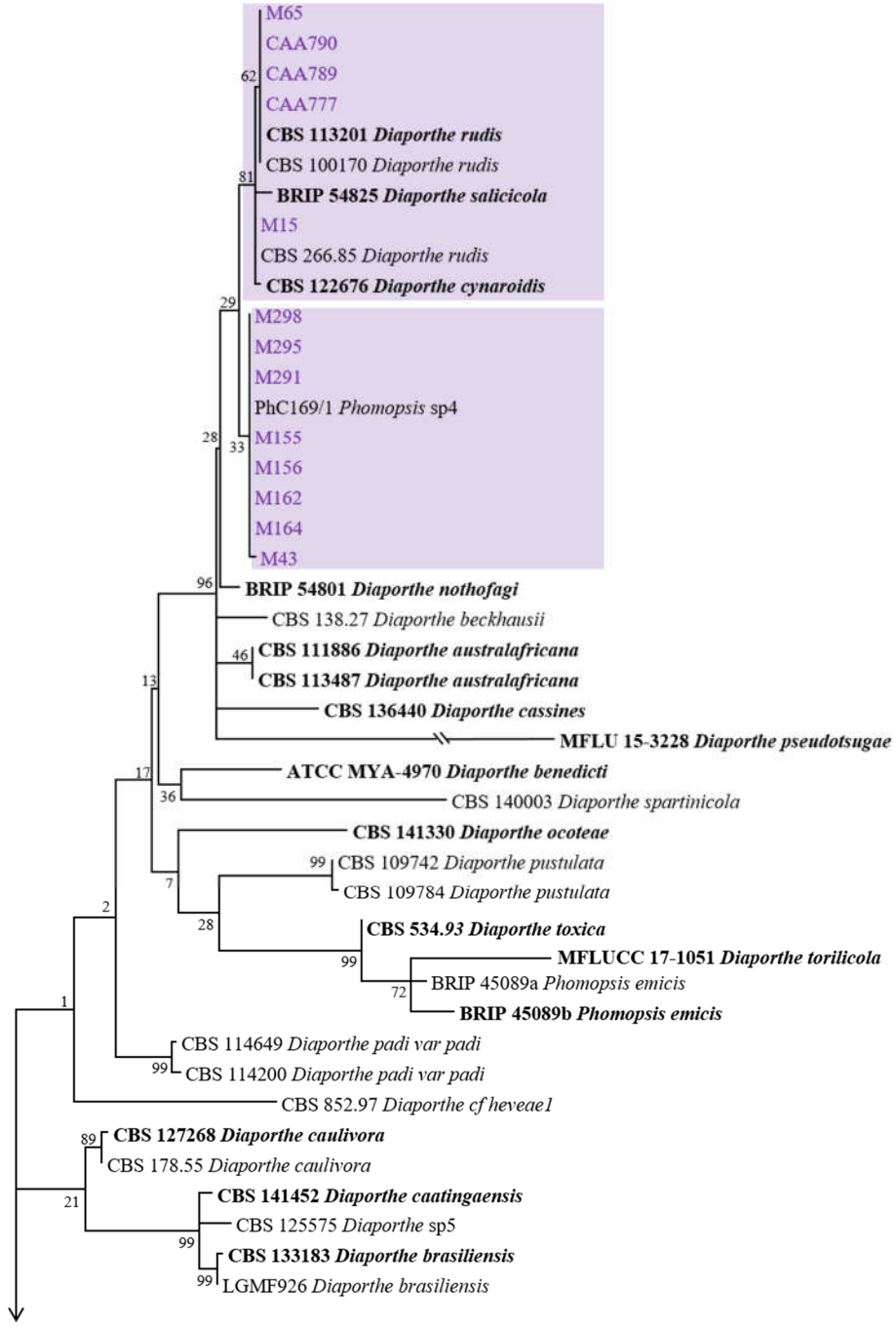

Figure 5 - ML phylogenetic tree of ITS region from *Diaporthe* species. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model. Bootstrap values are shown next to the branches. Ex-type isolates are given in **bold.**

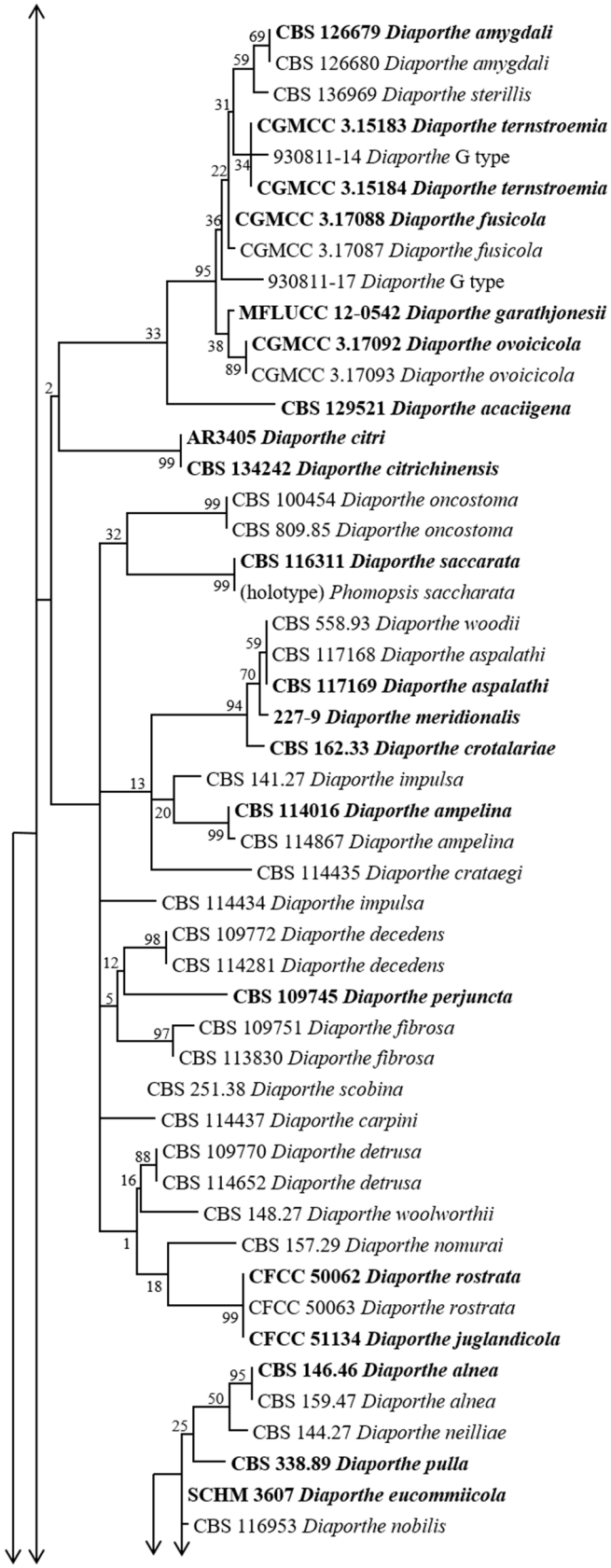

Figure 5 - continued

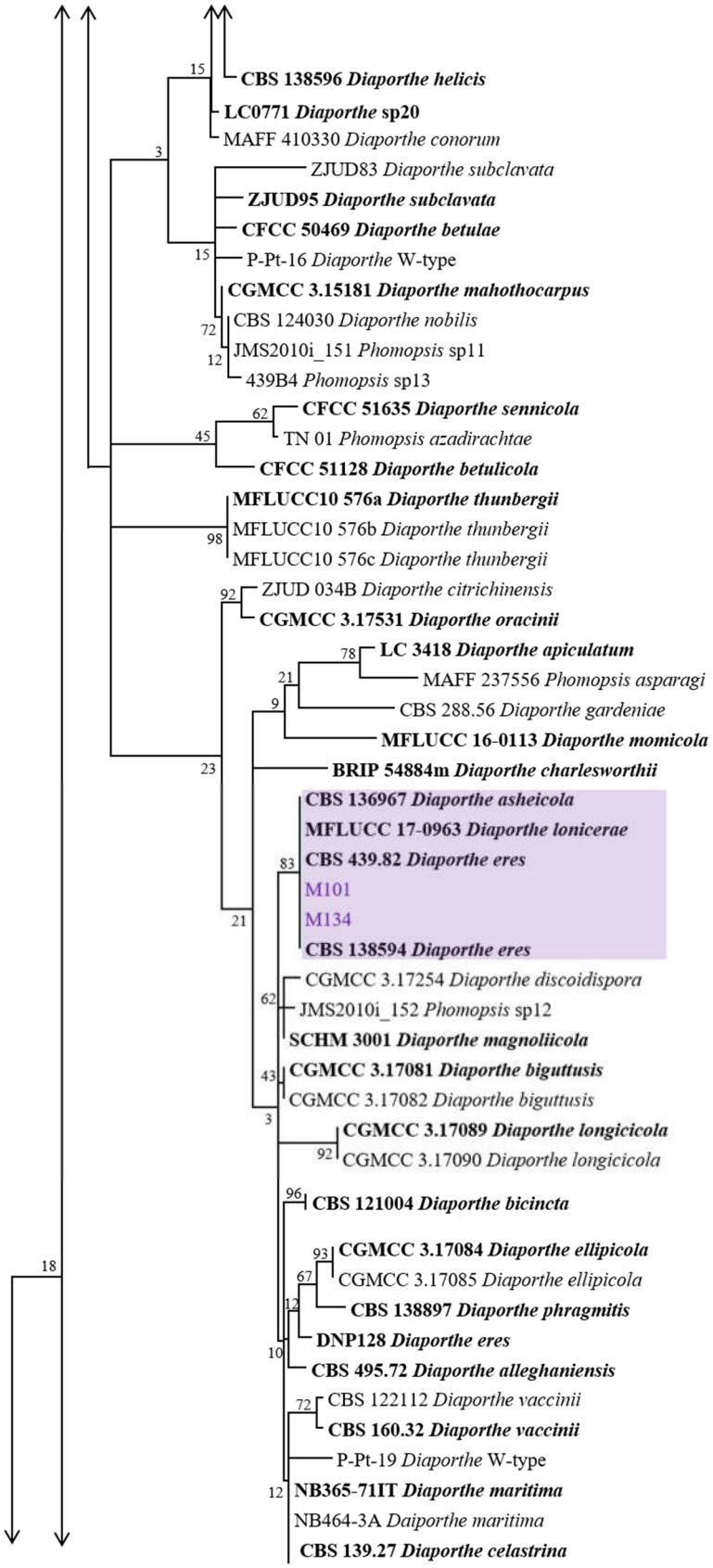

Figure 5 - continued

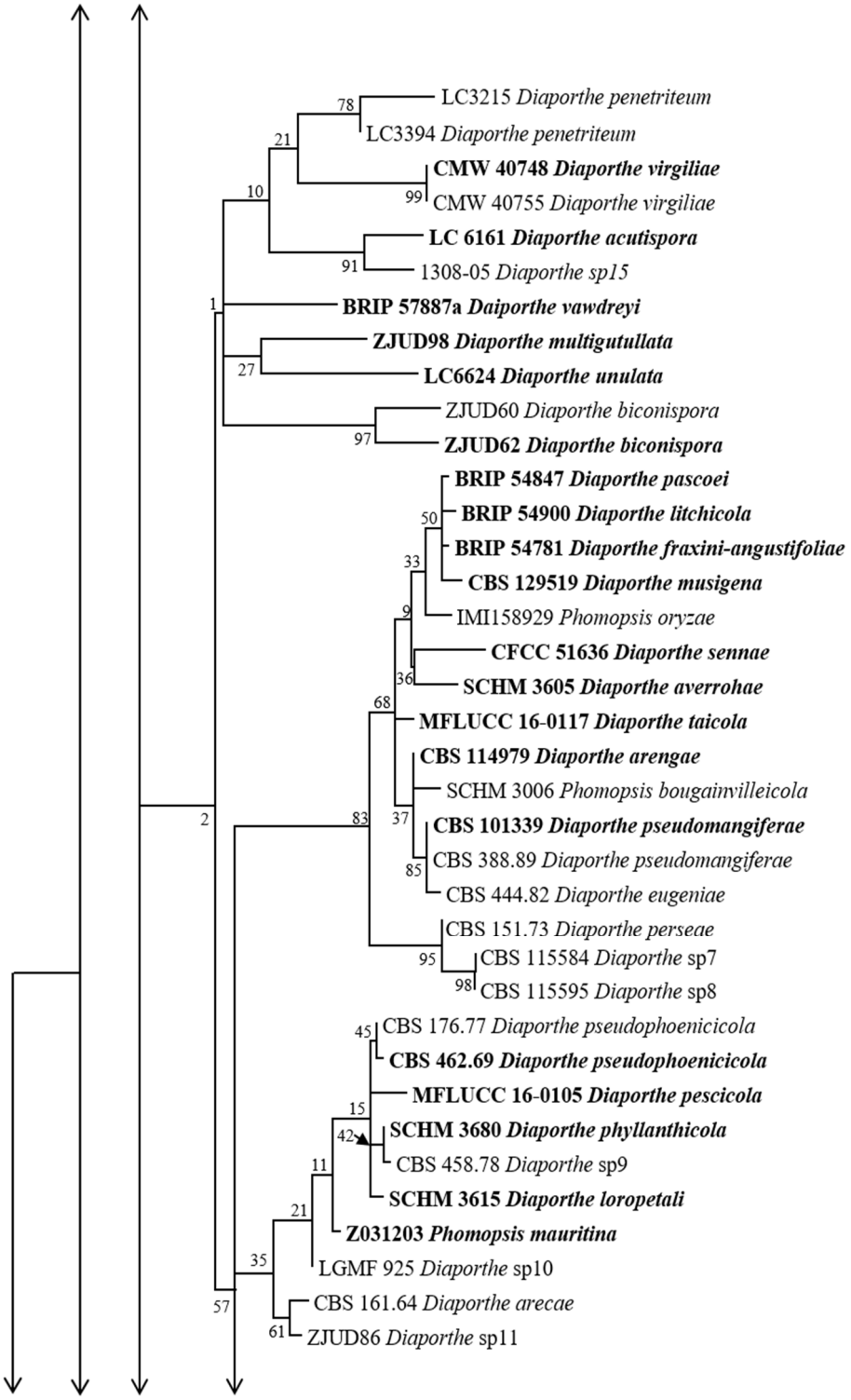

Figure 5 - continued

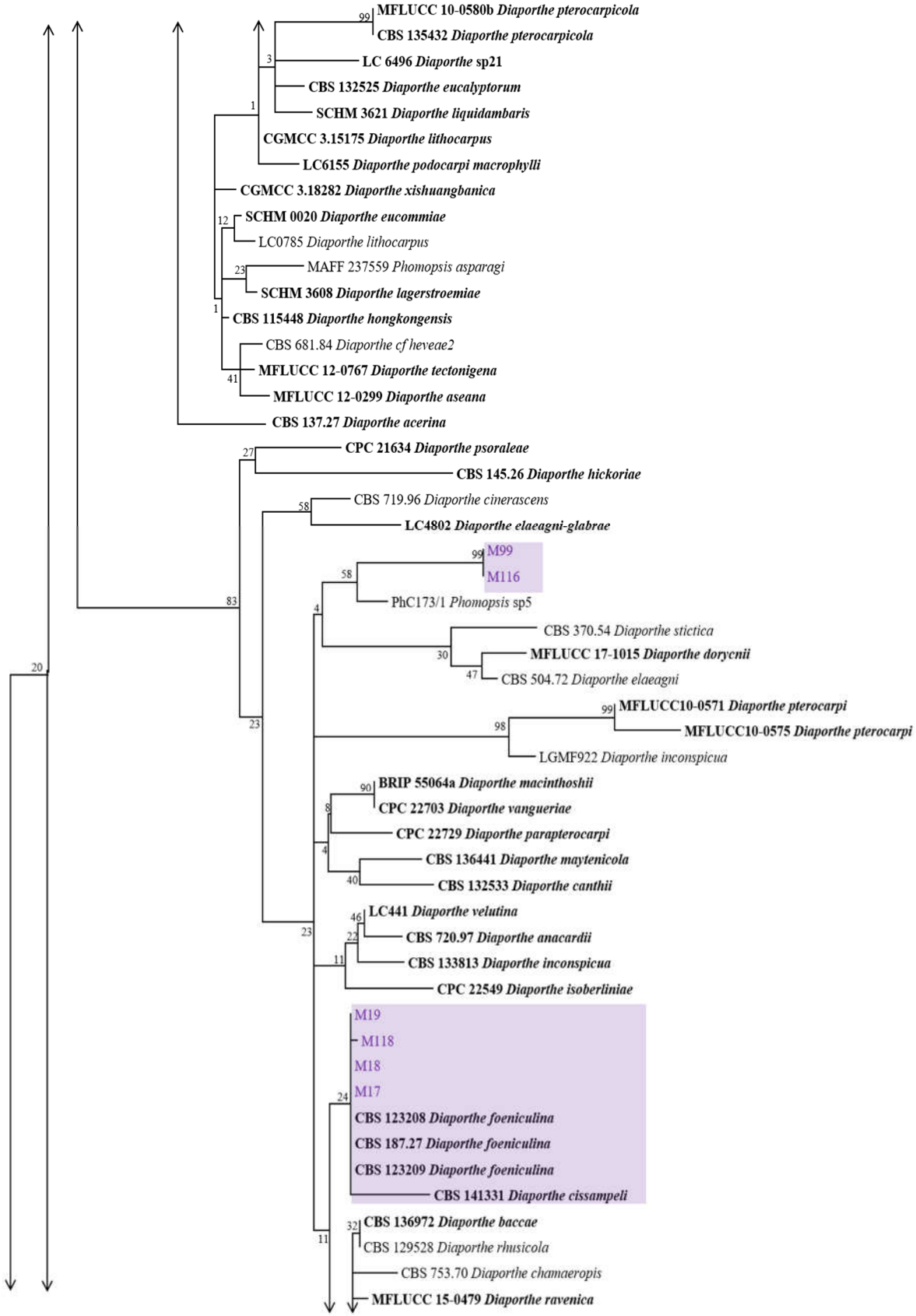

Figure 5 - continued

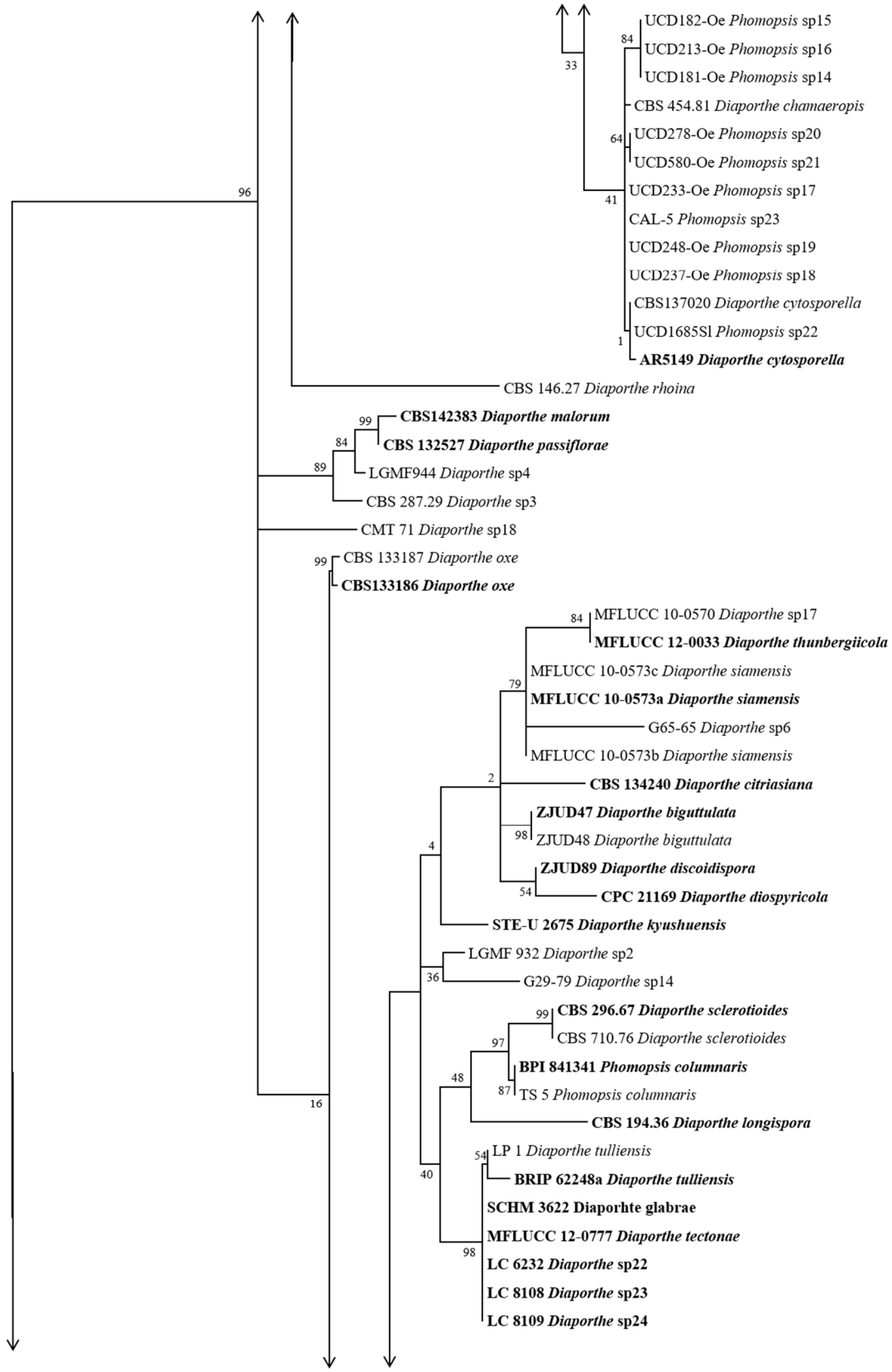

Figure 5 - continued

Figure 5 - continued

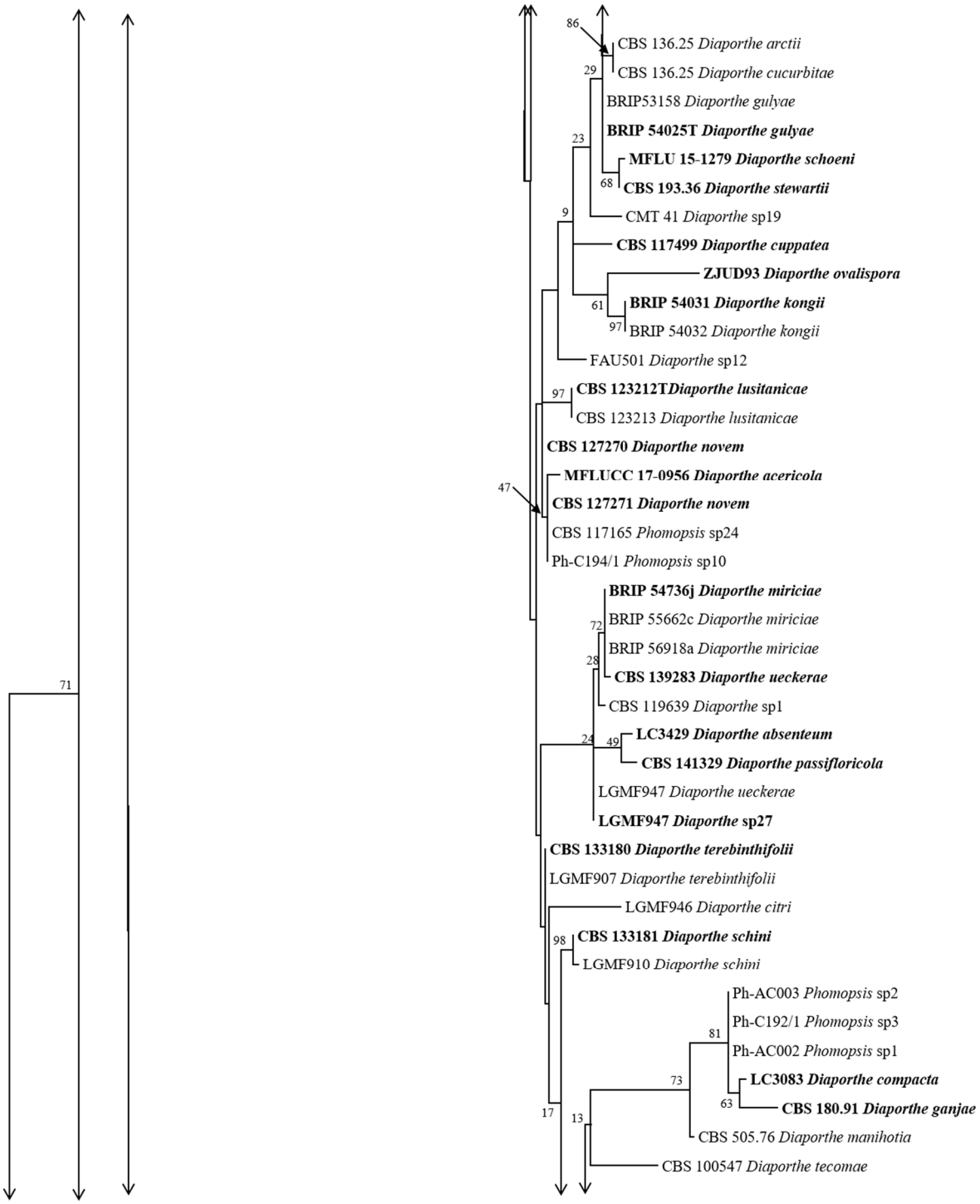

Figure 5 - continued

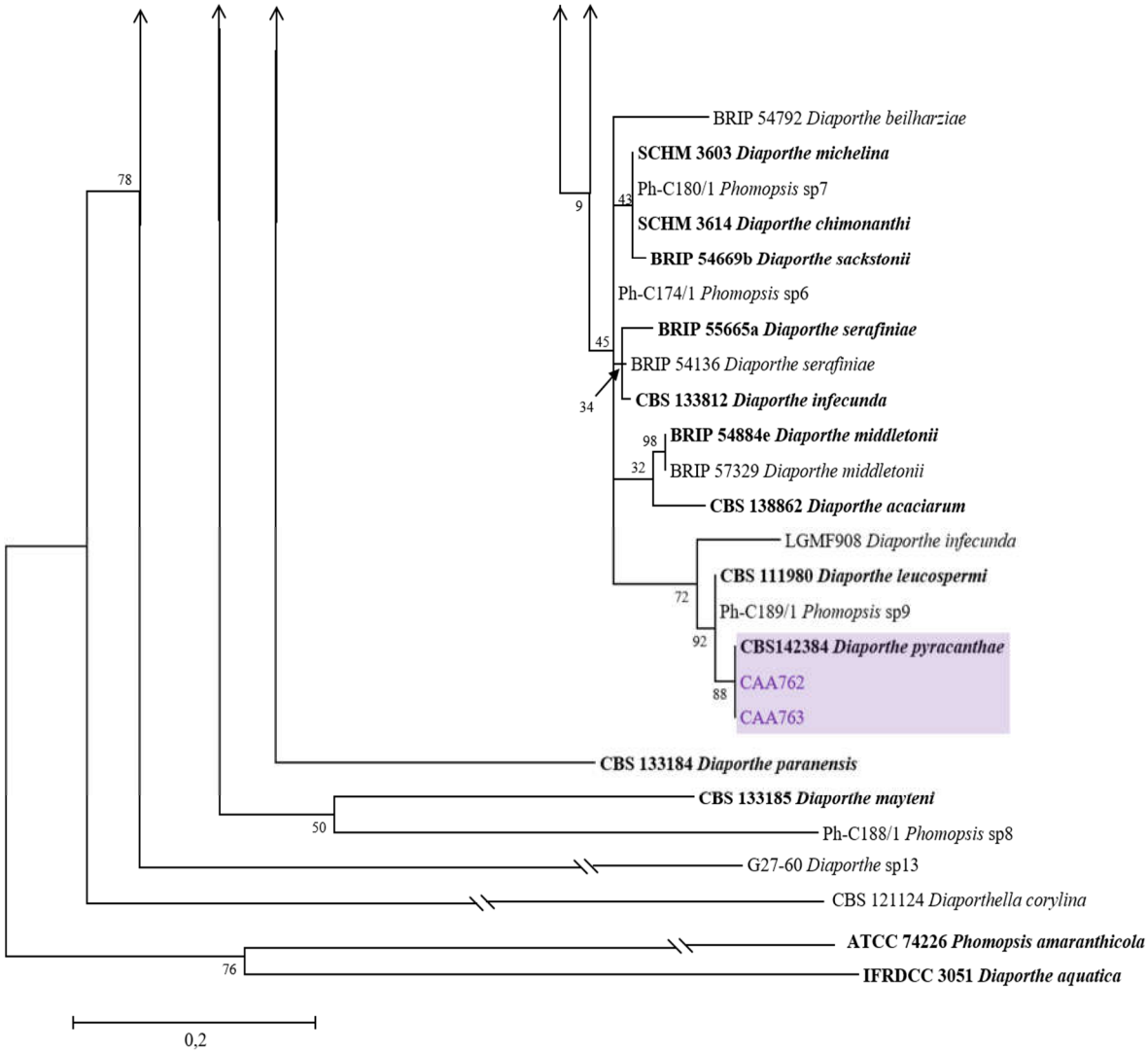

Figure 5 - continued

With 99 % bootstrap support, there is a clade containing 2 non-identified *Diaporthe* sp. 1 isolates (M99 and M116). The *Diaporthe foeniculina* clade, also containing a close relative *Diaporthe cissampeli,* is supported through a bootstrap value of 24 % and has grouped with 4 isolates (M17, M18, M19 and M118). CAA762 and CAA763 isolates, based on ITS, clustered with *Diaporthe pyracanthae* with 88 % of bootstrap support.

Since ITS analysis was not fully conclusive on the identity of the isolates, a multi-loci phylogenetic analysis with ITS*, tef1-α, tub, cal* and *his* was carried out for

a more accurate species separation (Figure 6). From the ITS ML tree, 13 representative isolates were selected.

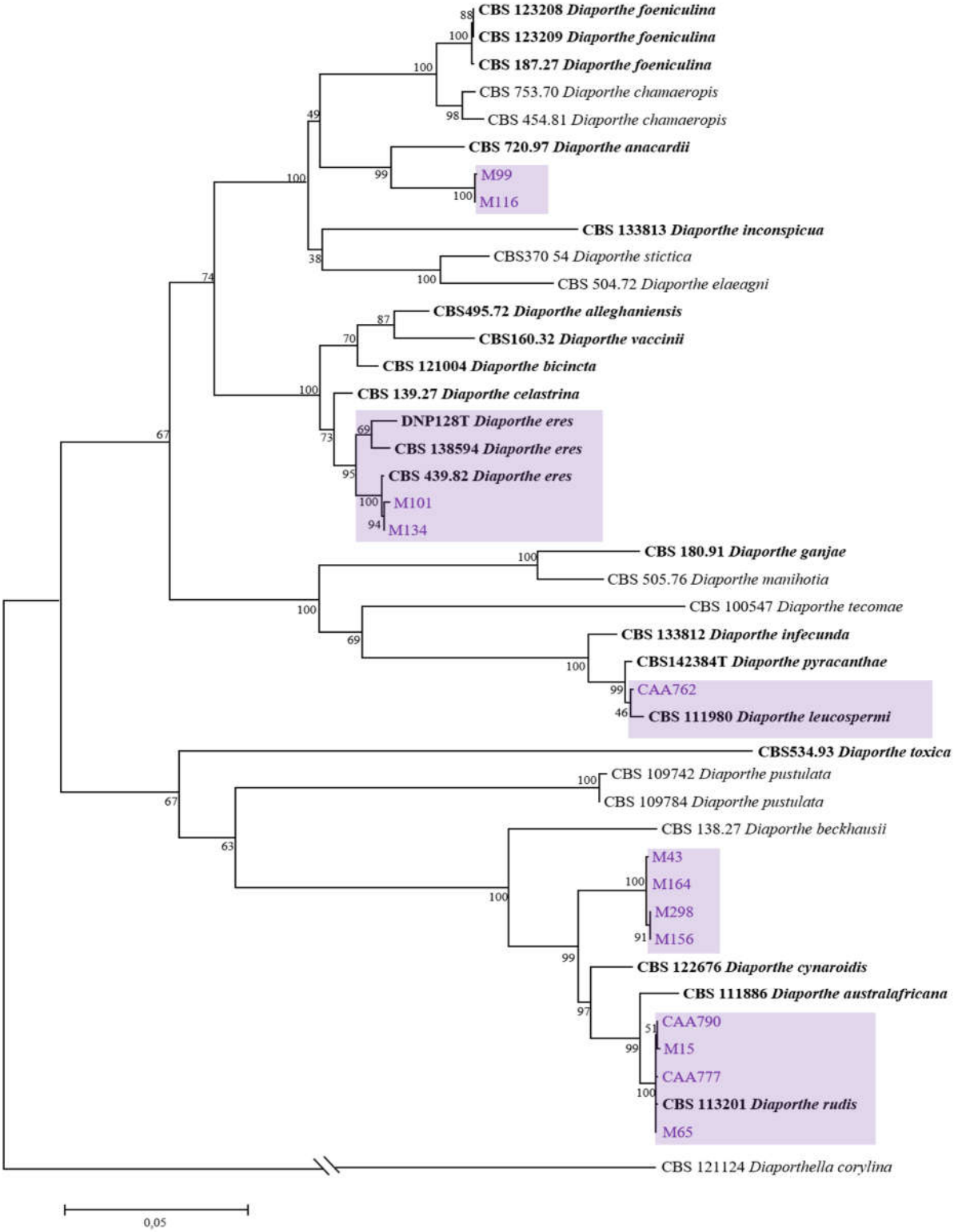

Figure 6 - ML phylogenetic tree obtained from combined analysis of ITS, *tef1-α, tub, cal* and *his* sequence dataset from species of *Diaporthe* based on the Tamura-Nei parameter model. Bootstrap values are shown next to the branches. Ex-type isolates are given **in bold.**

However, it was not possible to use none of the isolates M17, M18, M19 and M118 (identified as *Diaporthe foeniculina* based on ITS) in this analysis since *cal* gene was not determined. The analysis included 23 *Diaporthe* species phylogenetically closely related to our isolates, whose sequences for 5 loci were available.

From this multi-locus analysis, the 13 representative isolates chosen clustered within five clades, which correspond to five groups of species. Four isolates clustered in a clade containing the ex-type *Diaporthe rudis* (CBS 113201); two isolates grouped with *D. eres* (DNP128, CBS 128592 and CBS 439.82); one isolate clustered with *D. leucospermi* ex-type (CBS 111980) and the remaining isolates formed separate clades which correspond to two potentially novel species The clade containing the isolates M99 and M116 is well represented with a bootstrap support of 100 % and thus representative of a single new *Diaporthe* species (*Diaporthe* sp. 1). Although the isolates M298 and M156 are phylogenetically closely related to M43 and M164 with a bootstrap support of 100 %, M298 and M156 clustered in a sub-clade supported by a value of 91 %. To better understand this separation, the nucleotide positions of 5 loci were compared (Table 9).

Table 9 - Nucleotide differences between M43, M156, M164, M298. Shared polymorphisms are highlighted in grey.

Locus		**Isolates**			
		M43	M164	M156	M298
ITS (548 bp)	93	T	C	C	C
	100	G	G	G	-
tef1-α (317 bp)	-				
tub (399 bp)	13	C	C	G	G
his (440 bp)	-				
cal (458 bp)	-				

From this comparison, only 1 base in the *tub* locus seem to separate M156 and M298 from M43 and M164. It was not found any differences in the *tef1-α*, *his* and *cal* sequences from the isolates mentioned above. So, these 4 isolates are

representatives of a single new *Diaporthe* species (*Diaporthe* sp. 2). Yet in this phylogenetic analysis, the representative isolate CAA762 clustered with *D. leucospermi* that formed a sub-clade with a bootstrap support of 46 % within the *D. pyracanthae* clade. However, such result is not in concordance with the ITS phylogenetic analysis where it clusters with *D. pyracanthae*. Although *D. pyracanthae* is phylogenetically related to *D. leucospermi*, the species differ in several nucleotide positions as shown by Santos et al. (2017). Since the isolate CAA762 seem to cluster with either one or the other species, nucleotide sequences of this isolate were compared with the ones from *D. pyracanthae* and *D. leucospermi* (Table 10).

Table 10 - Nucleotide differences between *Diaporthe leucospermi*, *D. pyracanthae* and CAA762. Shared polymorphisms are highlighted in grey.

Locus		**Isolates**		
		D. leucospermi	*D. pyracanthae*	CAA762
ITS (537 bp)	61	C	T	T
	450	T	C	C
	467	T	C	C
tef1-α (332 bp)	16	C	T	T
	208	C	C	T
	213	T	T	A
tub (422 bp)	27	T	C	C
	45	A	G	A
	89	T	C	T
	161	T	C	T
	339	T	C	T
	347	T	C	T
his (457 bp)	188	G	A	A
	189	G	A	A
cal (493 bp)	-			

A comparison of ITS, *tef1-α*, *tub*, *cal* and *his* loci sequences of isolate CAA762 with *D. leucospermi* and *D. pyracanthae* showed that there are two unique polymorphisms in the sequences of *tef1-α* locus from isolate CAA762. Yet, this *Diaporthe* isolate shares polymorphisms in the sequences of ITS and *his* with *D. pyracanthae*; and in the sequence of *tub* with *D. leucospermi* (although it

shares one unique polymorphism with *D. pyracanthae* as well). No nucleotide differences were observed in the *cal* sequences.

Considering the uncertain molecular identification of the isolate CAA762 regarding its position in the phylogenetic trees as well as its nucleotide differences, the isolate is representative of a putative novel *Diaporthe* species (*Diaporthe* sp. 3).

5.4. Mating-type assay

The mating strategy was determined for 17 *Diaporthe* isolates (8 of them are representatives of potential novel species) (Table 11). From all the tested isolates, 9 were homothallic and 7 were heterothallic. Within *Diaporthe foeniculina* isolates, both mating types were identified with MAT1-2-1 (M118) and MAT1-1-1 genes (M17).

For 4 *Diaporthe* sp.2 (M43, M156, M164 and M298) homothalism was detected. Other 2 *Diaporthe* sp.1 isolates (M99 and M116) were identified as heterothallic once only MAT1-1-1 gene was detected. Two *Diaporthe rudis* isolates (M65 and M15) were also identified as homothallic containing both mating genes in the genome. CAA777, CAA789 and CAA790, identified as *Diaporthe rudis* were also classified as homothallic.

Table 11 – Mating types from *Diaporthe* isolates. (Note: ND – not determined).

Isolates		Mating types	
		MAT1	MAT2
Diaporthe rudis	M15	+	+
Diaporthe rudis	M65	+	+
Diaporthe foeniculina	M17	+	-
Diaporthe foeniculina	M118	-	+
Diaporthe sp. 1	M116	+	-
Diaporthe sp. 1	M99	+	-
Diaporthe eres	M101	+	-
Diaporthe eres	M134	+	-
Diaporthe sp. 2	M43	+	+
Diaporthe sp. 2	M156	+	+
Diaporthe sp. 2	M164	+	+
Diaporthe sp. 2	M298	+	+
Diaporthe rudis	CAA777	+	+
Diaporthe rudis	CAA789	+	+
Diaporthe rudis	CAA790	+	+
Diaporthe sp. 3	CAA762	ND	+
Diaporthe sp. 3	CAA763	ND	+

The 2 *Diaporthe eres* isolates were identified as heterothallic since only MAT1-1-1 gene was present. *Diaporthe* sp. 3 isolates CAA762 and CAA763 contain the MAT1-2-1 gene, but the MAT1-1-1 could not be determined.

5.5. Pathogenicity trials

For the pathogenicity tests, from the 10 isolates tested, 9 replicates per isolate were used to inoculate a total of 90 blueberry plants (cultivar *Bluecrop*). After 2 months, the Parafilm® was removed and brown external lesions were observed at the fungal inoculation sites. No internal lesions were observed on the control plants.

All healthy blueberry stems and branches inoculated with *Neofusicoccum parvum* (isolate M97) species displayed the most aggressive symptoms, some of them, 13 days after inoculation (Figure 7). The symptoms included brownish lesions, extensive outer epidermis and inner bark discoloration and stem blight which led to the leaves fall and consequently death of the plant.

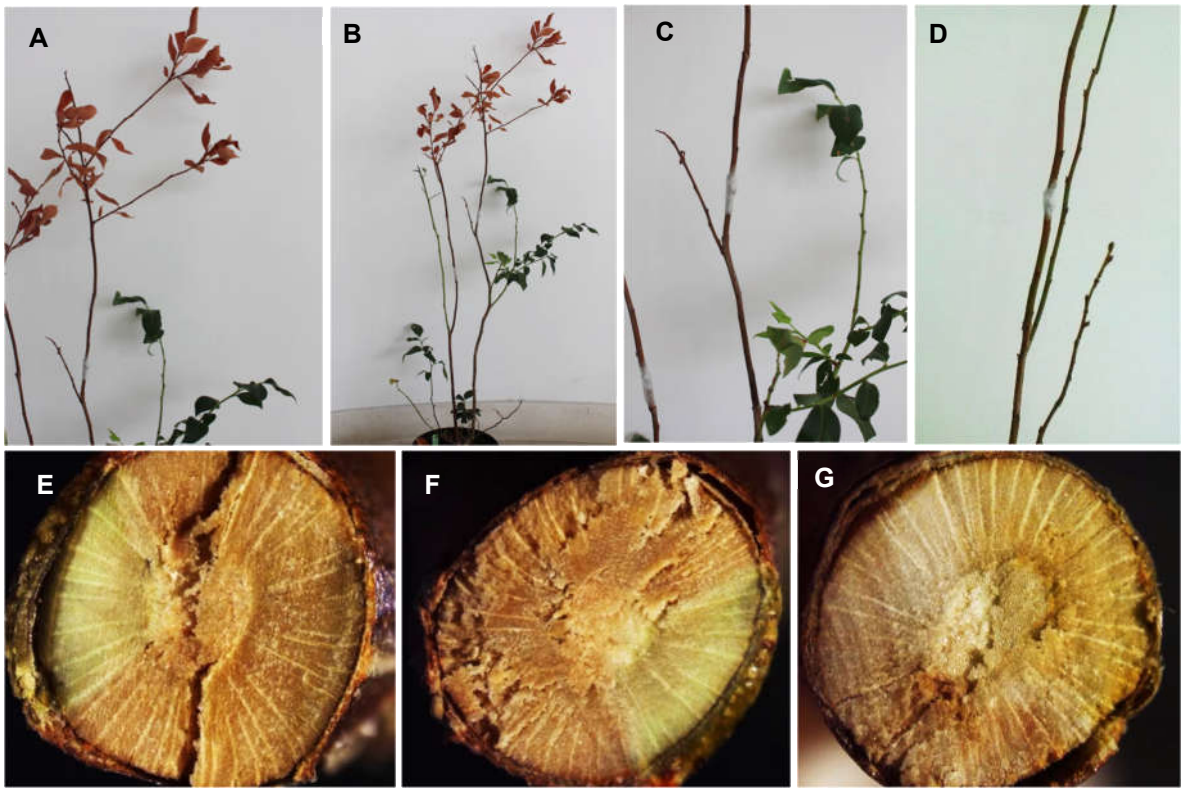

Figure 7 – Symptoms on *Vaccinium corymbosum* (cv. *Bluecrop*) caused by *Neofusicoccum parvum* inoculation. (**A, B**) – drought of leaves and stems. (**C**) – inoculation point and stem drought. (**D**) – death of plant. (**E, F, G**) – stem displaying a progressive discoloration of the vascular tissues.

Plants inoculated with *Diaporthe* sp. 3 (isolate CAA762) started to exhibit yellow to brown leaves 15 days after inoculation.

The rest of inoculated blueberries plants showed healthy leaves and did not exhibit any other symptom but small lesions confined to the inoculation point.

The external lesions were mainly discoloration of the tissues that ranged from yellow to brown and in some cases, cankers were visible as reaction to the infection. The internal lesions were characterised by brownish colour of the inner vascular tissues, and the lengths varied depending on the species inoculated.

Neofusicoccum parvum isolate collected in this study caused significantly larger external and internal necrotic lesions and thus considered the most virulent among *Neofusicoccum* species ($F_{3,32}$ = 3.9227; $p<0.05$) (Figure 8). From the 9 replicates, 3 of them caused the death of plants. *Neofusicoccum eucalyptorum* caused external necrotic lesions similar in length to those of *N. australe* and *Botryosphaeria dothidea* ($F_{3,32}$ = 3.9227; $p<0.05$).

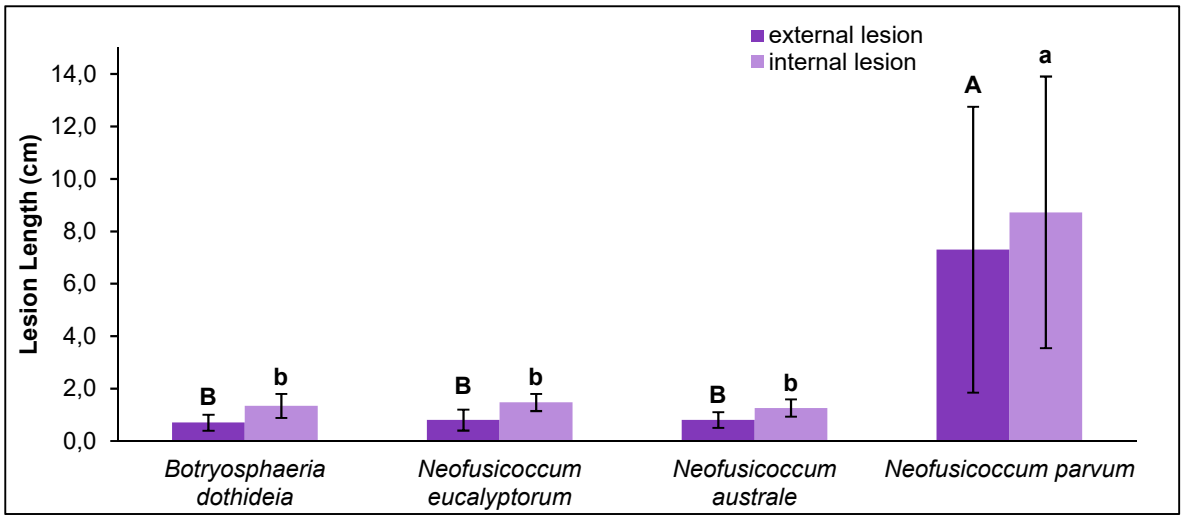

Figure 8 - Lesion lengths on blueberry stems and branches caused by *Botryosphaeriaceae* species after 2 months. The vertical lines indicate standard deviations. Bars with the same letter are not significantly different.

Among *Diaporthe* isolates, *Diaporthe* sp. 3 (CAA762) showed significantly higher internal lesion length than the rest of the isolates, and so was regarded as the most aggressive *Diaporthe* species ($F_{5,48}$ = 4.4311; $p<0.05$) (Figure 9). The external lesion lengths were significantly different between *D. rudis* that caused the smallest lesions and *D. eres* that showed the highest ones ($F_{5,48}$ = 4.4311; $p<0.05$).

Overall, inoculations with *Diaporthe* species exhibited more superficial lesions, while *Neofusicoccum* and *Botryosphaeria* inoculations caused deeper lesions in the vascular tissues.

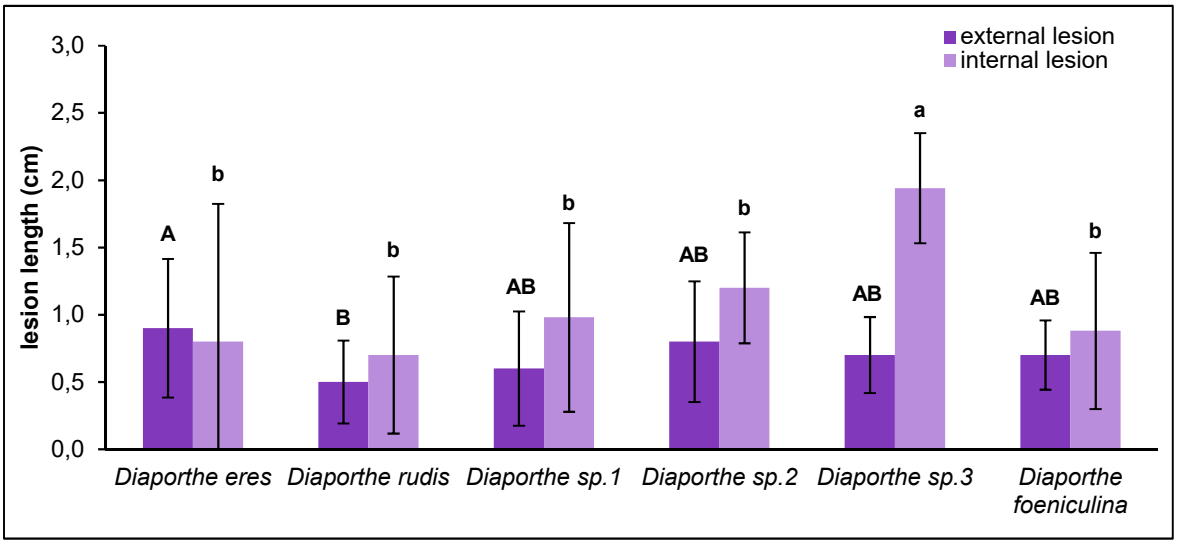

Figure 9 - Lesion lengths on blueberry stems and branches caused by *Diaporthe* species after 2 months. The vertical lines indicate standard deviations. Bars with the same letter are not significantly different.

The pathogens were re-isolated from symptomatic inner bark of all inoculated stems and branches and identified by morphological observation, thus confirming Koch's postulates. No isolates were obtained from the controls.

5.6. Effect of temperature on mycelial growth

Temperature growth studies were made for 7 isolates of putative new *Diaporthe* species as inferred by phylogenetic analysis. The colony diameters were measured daily until the colony reached the edges of the plate (Figure 10).

Overall all the isolates grew better at 25°C. In the first 3 days, *Diaporthe* sp. 1 isolates M99 and M116 showed a better growth at 30°C than at 25°C. *Diaporthe* sp. 2 isolates M43, M156, M298 and M164 presented a slow growth at 30°C and 35°C. The isolates M43 and M164 showed a higher growth at 30°C than 10°C. However, after day 5, the growth at 10°C surpassed the one at 30°C.

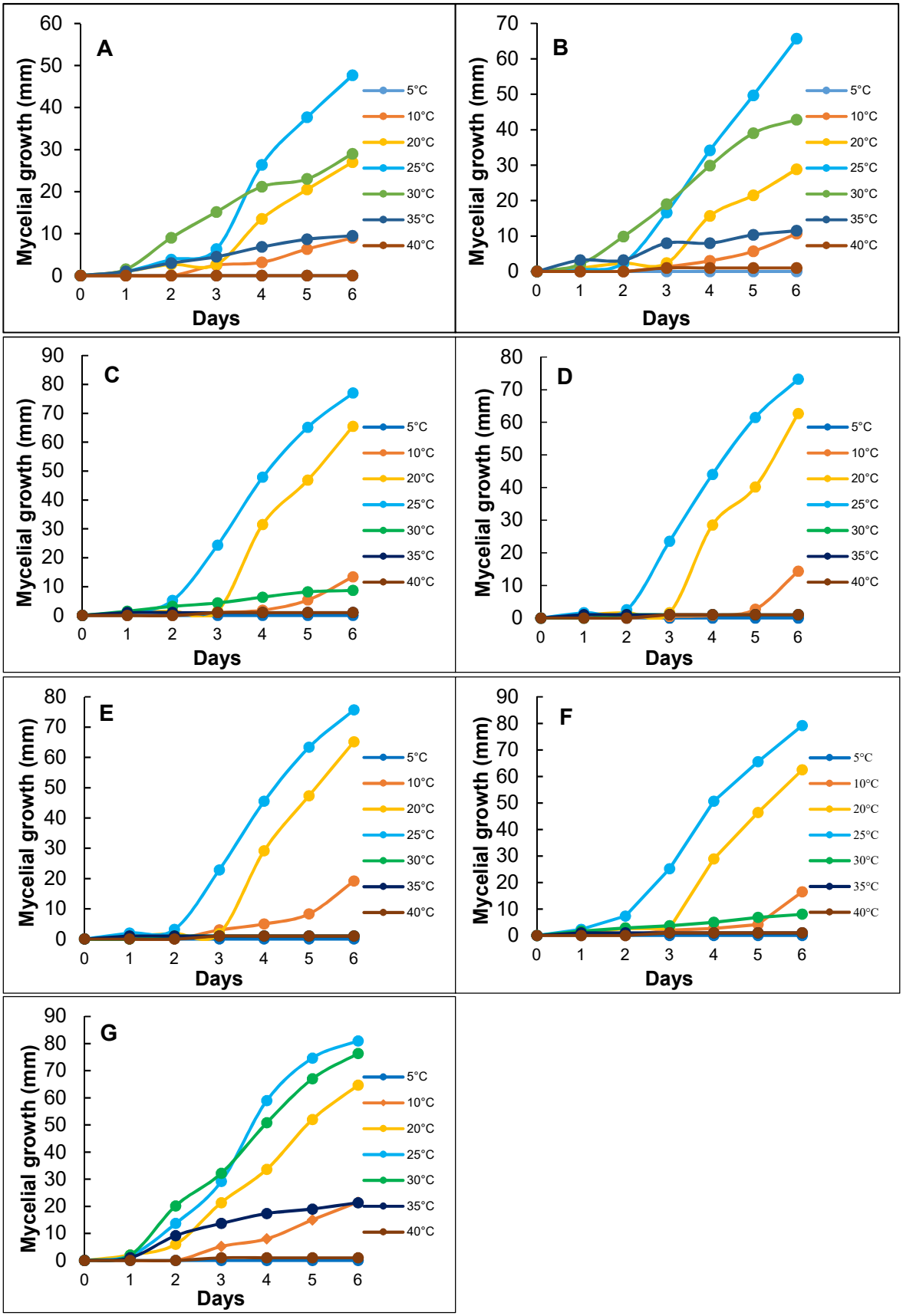

Figure 10 – Mycelial growth curves of *Diaporthe* sp. 1 isolates M99 **(A)** and M116 **(B)**; *Diaporthe* sp. 2 isolates M43 **(C)**, M156 **(D)**, M164 **(E)** and M298 **(F)**; *Diaporthe* sp. 3 isolate CAA762 **(G)**.

After 2 days of the incubation at 30°C, the isolates M164 and M156 presented a brown pigmentation in the medium. At 35°C, apart from these, M43 and M298 also displayed such colour in the medium (Figure 11). The isolate CAA762 was the one with the faster mycelial growth at all temperatures tested. At 5°C, none of the isolates was able to grow. However, as soon as the plates were taken out the incubator and placed at room temperature, re-growth was observed. Forty degrees temperature was lethal to most of the isolates since when returning to room temperature they did not recover. Inversely, *Diaporthe* sp. 3 isolate CAA762 was the one that did recover after being under such temperature.

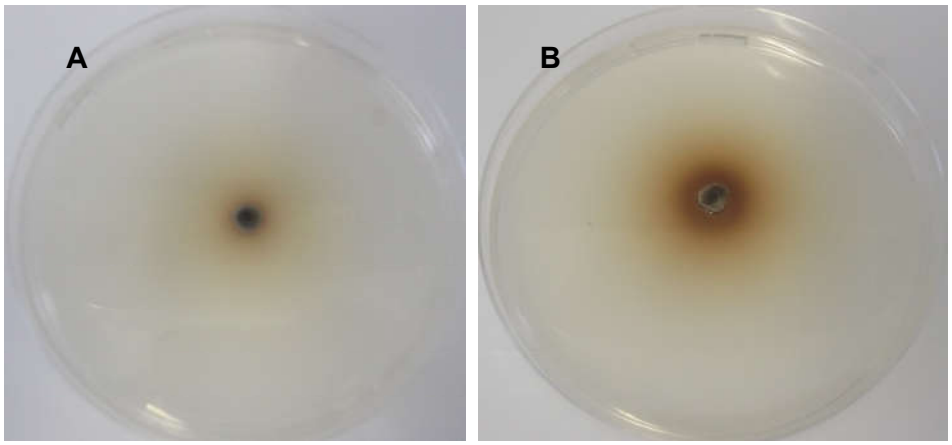

Figure 11 – Isolates M43 (**A**) and M298 (**B**) displaying a brown pigmentation 2 days after incubation at 35°C.

5.7. Taxonomy

For all monosporic cultures, we recorded the pycnidia and conidia features. Produced pycnidia from the monosporic culture procedure, were dissected and the conidia were mounted in 100 % lactic acid. Observations were made with the Nikon SMZ1500 stereomicroscope (Nikon, Japan) for pycnidia and Nikon eclipse 80i microscope (Nikon, Japan) for conidia. All photographs from pycnidia were captured with a Digital Sight DS-Fi1 camera and from spores with a Nikon Digital Sight DS-Ri1 camera (Nikon, Japan) (Figures 12 to 18).

Figure 12 – *Neofusicoccum eucalyptorum* isolate M85. **Left**: Pycnidia and brown to grey conidial cirrhus on fennel twigs. **Right:** conidia

Figure 13 – *Neofusicoccum parvum* isolate M22. **Left**: Pycnidia and yellow conidial cirrhus on pine needles. **Right:** conidia.

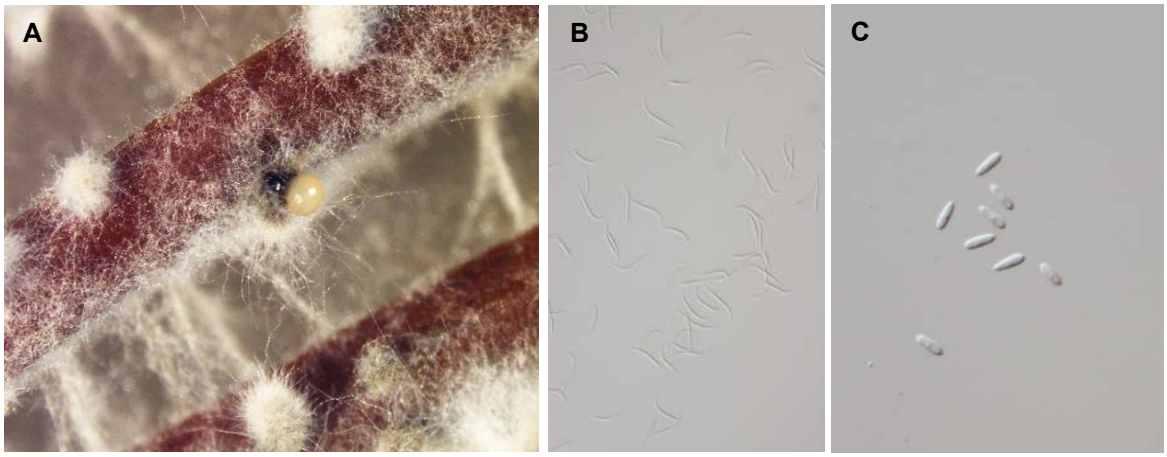

Figure 14 – *Diaporthe foeniculina* isolate M18. **(A)** Pycnidia and yellow to orange conidial cirrhus on pine needles. **(B)** beta conidia. **(C)** alpha conidia.

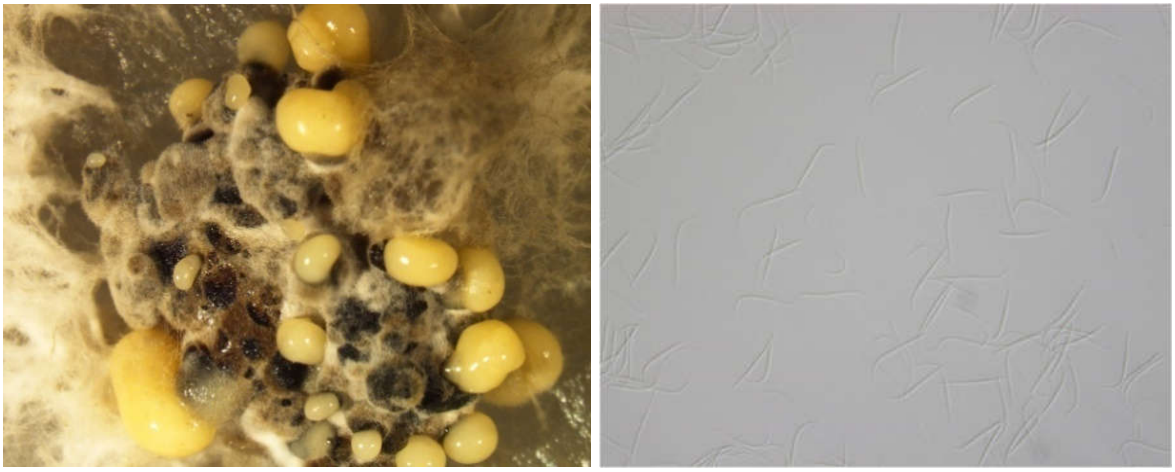

Figure 15 – *Diaporthe eres* isolate M134. **Left**: Pycnidia and yellow conidial cirrhus on ½ strength PDA medium. **Right:** beta conidia.

***Diaporthe* sp. 1** S. Hilário, L. Santos & A. Alves*, sp. nov.*

Diaporthe sp. 1 representative isolate M99 has superficial pycnidia and exuds a yellow conidial cirrhus (Figure 16). Alpha conidia on pine needles on WA medium (mean ± S.D. = 6.1 ± 0.7 × 2.7 ± 0.5 µm, n = 100), and on ½ PDA (mean ± S.D. = 7.1 ± 0.8 × 2.7 ± 0.5 µm, n = 100). Alpha conidia on fennel twigs on ½ PDA (mean ± S.D. = 6.5 ± 0.9 × 2.8 ± 0.6 µm, n = 100). Beta and gamma conidia were not observed.

Culture characteristics – Colonies spreading with sparse aerial mycelium with a pale brown concentric zone and covering a Petri dish in 7 days at 25 ºC. The colony grew better at 30°C than 25°C in the first 3 days. After the fourth day, the growth was higher at 25°C. It did not grow at 5°C nor at 40°C. At day 6, the mycelial growth at 10°C overlapped the one at 35°C (Figure 10A).

***Diaporthe* sp. 2** S. Hilário, L. Santos & A. Alves*, sp. nov.*

Diaporthe sp. 2 representative isolate M156 shows superficial pale brown pycnidia (Figure 17). Alpha conidia on fennel twigs on ½ PDA (mean ± S.D. = 6.9 ± 0.9 × 2.7 ± 0.5 µm, n = 100). Alpha conidia on pine needles on PDA ½ (mean ± S.D. = 7 ± 0.8 × 2.8 ± 0.5 µm, n = 50). Beta and gamma conidia were not observed.

Culture characteristics – Colonies spreading with sparse aerial mycelium with pale to dark grey concentric zones and covering a Petri dish in 7 days at 25

ºC. The colony grew better at 25 °C. Only after the 5th day, the culture started to exhibit some growth at 10°C (Figure 10D). At 30°C and 35°C, the culture did not grow but displayed a circular brown pigmentation (Figure 11).

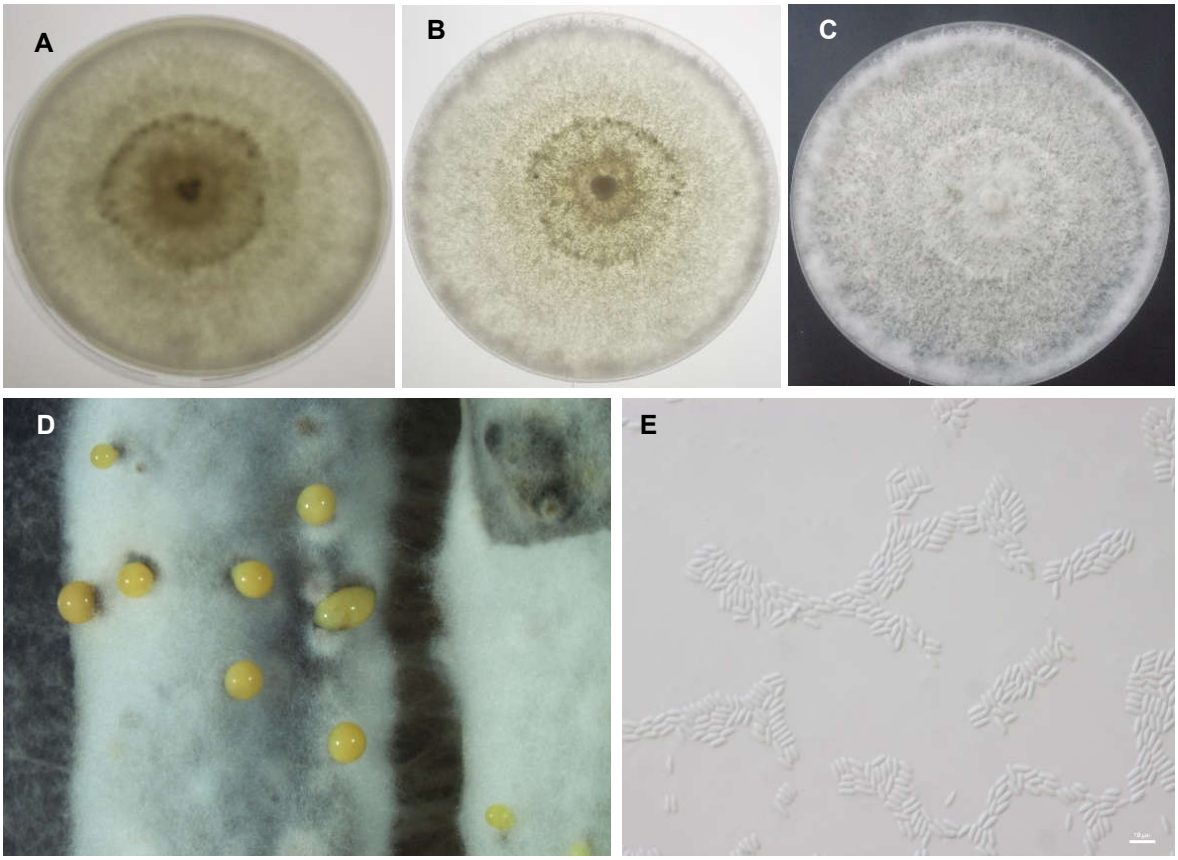

Figure 16 – *Diaporthe* sp. 1 isolate M99. **(A)** 7-day-old reverse culture surface on PDA at 25 ºC. **(B, C) 7-**day-old upper culture surface on PDA at 25ºC. **(D)** Pycnidia exuding a yellow conidial cirrhus on fennel twigs. **(E)** alpha conidia. Scale bar: 10 µm

***Diaporthe sp.* 3** S. Hilário, I. Amaral, L. Santos & A. Alves*, sp. nov.*

Diaporthe sp. 3 representative isolate CAA762 is morphologically identical to *Diaporthe leucospermi* and *Diaporthe pyracanthae*, showing superficial pycnidia, dark brown and exuding a creamy white conidial cirrhus (Figure 18). Alpha conidia on fennel twigs on WA medium (mean ± S.D. = 6.9 ± 0.8 × 3.0 ± 0.5 µm, n = 100). Beta conidia on fennel twigs on WA (mean ± S.D. = 23.1 ± 3.6 × 1.7 ± 0.4 µm, n = 50) and on PDA ½ (mean ± S.D. = 29.2 ± 3.8 × 1.5 ± 0.4 µm, n = 100). Gamma conidia were found rare.

Culture characteristics – White colonies spreading large with moderate aerial mycelium, yellow concentric zone, covering a Petri dish in 6 days at 25°C.

The colony grew better at 30°C than at 25°C in the first 3 days. After the fourth day, the growth was higher at 25°C. It grew better at 35 °C than at 10°C. However, at day 6, the growth at both temperatures was the same (Figure 10G) This isolate was the only that recovered after has been taken out from the 40°C incubator.

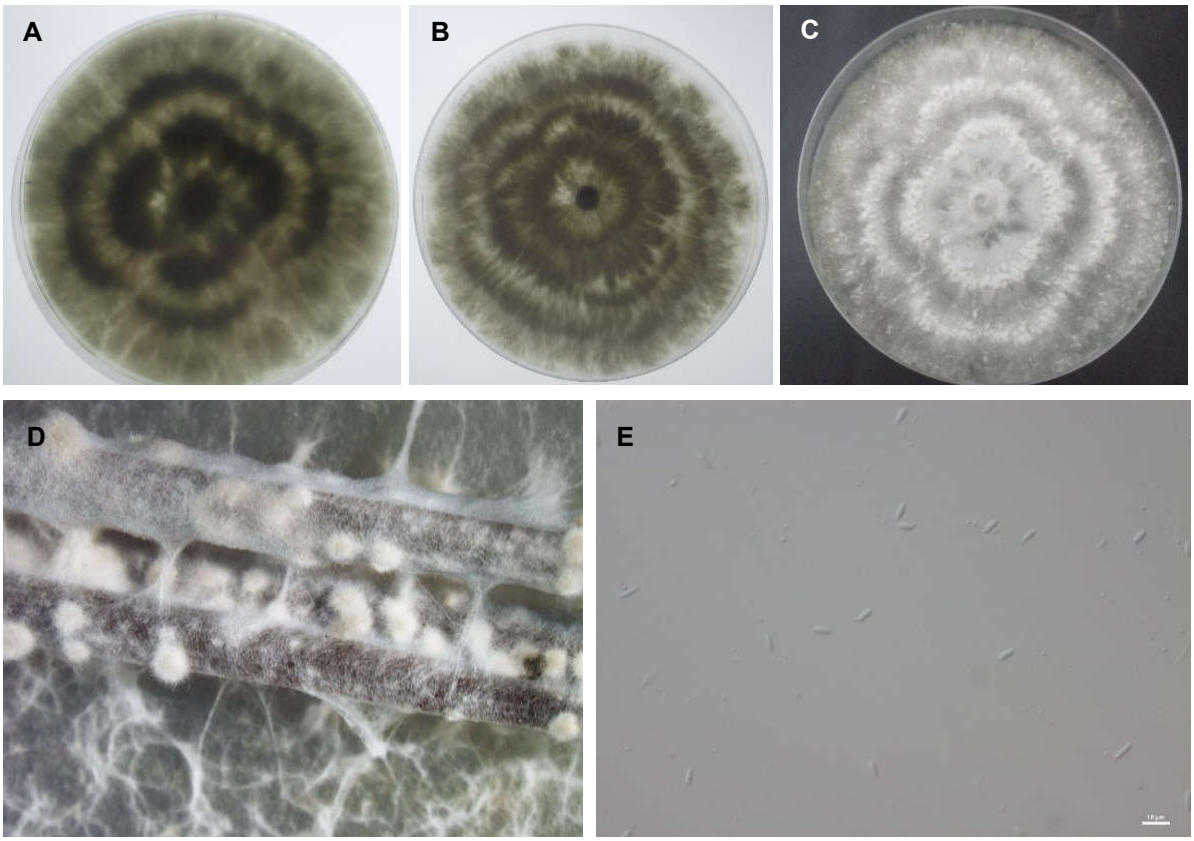

Figure 17 – *Diaporthe* sp. 2 isolate M156. **(A)** 7-day-old reverse culture surface on PDA at 25 ºC. **(B, C) 7-**day-old upper 7-day-old culture surface on PDA at 25ºC**. (D)** Pycnidia on pine needles. **(E)** alpha conidia. Scale bar: 10 µm

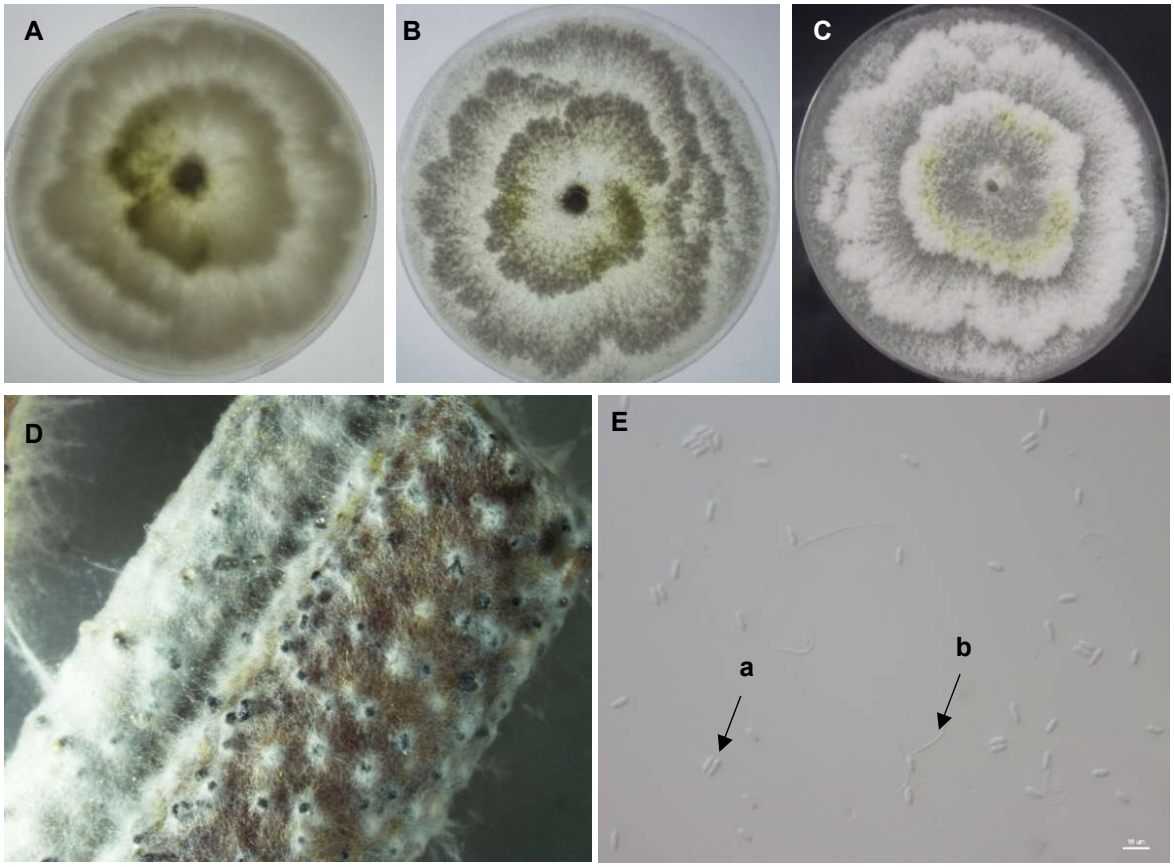

Figure 18 – *Diaporthe* sp. 3 isolate CAA762**. (A)** 7-day-old reverse culture surface on PDA at 25 ºC. **(B, C) 7-**day-old upper culture surface on PDA at 25ºC. **(D)** Pycnidia and white conidial cirrhus on fennel twigs**. (E)a** – alpha conidia; **(E)b** – beta conidia. Scale bar: 10 µm

6. DISCUSSION

The current study addresses the fungal diversity associated with blueberry plants (*Vaccinium corymbosum*) in plantations located in Ílhavo, Aveiro and Sever do Vouga, that occurred mostly during late spring and early summer (June and July) on several cultivars. This latter region is the major blueberry growing area of Portugal (Oliveira & Fonseca, 2007).

In Portugal, very little is known regarding the fungal diseases of *Vaccinium corymbosum*. However, *Armillaria* spp., *Fusarium* spp. and *Phytophtora* spp. are considered the most important pathogens occurring in the country (Chicau, 2015; Diogo et al., 2016; Madeira, 2016). Although Diogo et al. (2016) have shown the existence of *Botryosphaeriaceae* and *Diaporthe* species on blueberries in Portugal, no exhaustive studies have been carried out so far. Thus, a special emphasis was put on members of the *Botryosphaeriaceae* and *Diaporthaceae* (genus *Diaporthe*), due to their relevance as known plant pathogens, affecting commercial cultivated blueberry worldwide (Lombard et al., 2014; Xu et al., 2015).

All the isolates identified in this study, from the families *Botryosphaeriaceae* and *Diaporthaceae*, were previously reported on other host plants, suggesting that while some species appear to be host specific, many are not and can be found on diverse plant hosts (Gomes et al., 2013; Udayanga et al., 2011). Species of *Botryosphaeriaceae* and *Diaporthe* are similarly known as causing diseases on numerous hosts with economic importance such as: walnut, pistachio, avocado, conifers, blueberries, fennel, almond, eucalypts and grapevines (Alves et al., 2013; Barradas et al., 2016; Chen et al., 2014a; Chen et al., 2014b; Cinelli et al., 2015; Diogo et al., 2010; Guarnaccia et al., 2016; Lombard et al., 2014; Phillips et al., 2002; Sammonds et al., 2009; Santos & Phillips, 2009; Wright et al., 2012).

Botryosphaeria dothidea was the only representative of the genus found in this study (100 %). It is a plurivorous and widespread species causing damages on diverse hosts, such as shoot blight in *Arbutus unedo* and *Juglans regia*, cankers in *Eucalyptus* species (although rare) and *Vitis vinifera* and fruit rot in *Prunus domestica*, *Olea europaea* and *Malus domestica* (Moral et al., 2010; Pérez et al., 2008; Phillips, 2002; Phillips et al., 2005; Roca et al., 2013; Slippers et al., 2007; Úrbez-Torres, 2011; Xu et al., 2014). At present, *B. dothidea* has been

reported to cause not only stem blight, but also wilting of twigs, chlorosis and necrosis of leaves on individual branches, stem cankers and stem dieback on blueberries in Korea, China and USA (Choi, 2011; Milholland, 1972; Polashock & Kramer, 2006; Smith & Stanosz, 2001; Wright & Harmon, 2010; Yu et al., 2012).

Isolates identified as *B. dothidea* were obtained from both asymptomatic branches and symptomatic plant material, namely associated with dead branches, and branch dieback and canker. Once again, this study confirms the occurrence of *B. dothidea* on plant tissues as endophyte, a characteristic common to several members of the family (Osorio et al., 2017; Slippers et al., 2007). On symptomatic plants, *B. dothidea* commonly co-occurred with other species, namely *Neofusicoccum parvum* and *N. australe* and therefore it is not possible to know which(s) species are the causal agent of the observed symptoms.

Pathogenicity tests carried out revealed that *B. dothidea* can cause lesions on stems and branches when inoculated, although it was not the most aggressive of the species tested (Figure 8). Although Milholland (1972) and Xu et al. (2015) stated that *B. dothidea* is the most prevalent species on blueberry-growing regions and the main causal agent of stem blight, in the present study, this species did not show to be neither the most dominant nor the most aggressive. Nevertheless, this study establishes the first confirmed report of *B. dothidea* on *Vaccinium corymbosum* in Portugal.

Botryosphaeria corticis, considered a serious pathogen on blueberries in the Southeastern United States, has been reported only in the USA as causing stem canker (Milholland, 1972; MSU, 2016; Phillips et al., 2006; Wright & Harmon, 2010). Although host specificity is now regarded as infrequent in *Botryosphaeriaceae* (Phillips et al., 2013)*, B. corticis* does show specificity on this host. So far, it has not been reported from any other host apart from *Vaccinium*. While studies by Phillips et al. (2006) allowed to distinguish *B. dothidea* and *B. corticis* through molecular and morphological characterisation, pathogenicity tests run by Espinoza et al. (2009) showed that symptoms caused by both species are similar.

In the present study, *B. corticis* was not recovered from any of the sample material we surveyed. However, considering the low number of samples used

along with a restricted sampling area, we cannot exclude its presence in the country. Tennakoon et al. (2017) have shown that nursery plants might represent a source of inoculum for the introduction of the pathogens to blueberry plantations. Considering that blueberry was introduced primarily from the USA, it is interesting to highlight that *B. corticis* was not found outside the USA yet (Farr & Rossman, 2017). Nevertheless, although Phillips et al. (2006) described *B. corticis* as a distinct species from *B. dothidea*, the absence of *B. corticis* in sampled material outside USA seems strange but it may be possibly linked with an incorrect identification since *B. dothidea* has been the most widely accepted pathogen name (Xu et al., 2015).

Species of *Neofusicoccum* occur in almost all type of woody plant hosts, including economically important fruit crops. They are known to infect wounded and non-wounded tissues, representing a risk to susceptible plants (Lopes et al*.,* 2016; Tenakoon et al., 2017a). In this study, isolates of *Neofusicoccum* were the most abundant in the collection analysed (35 %). These isolates where identified as belonging to 3 different species namely *N. australe*, *N eucalyptorum* and *N. parvum*.

Among the *Neofusicoccum* species causing stem dieback on *V. corymbosum*, *Neofusicoccum arbuti*, *N. australe* and *N. parvum* were found in Chile, Spain and New Zealand, indicating that *Botryosphaeriaceae* species are common pathogens of blueberries (Castillo et al., 2013; Espinoza et al., 2009; Tennakoon et al., 2017b). Additionally, in Chile, the species *N. nonquaesitum* was detected in branches of *V. corymbosum* cvs. *Brigitta* and *Elliott,* (Pérez et al., 2014). However, this species along with *N. arbuti* were not found in the present study. Also, Yu et al. (2013) showed that symptoms caused by *N. vitifusiforme* are similar to those caused by *N. parvum* and it was confirmed as a pathogen of blueberries in China (Kong et al., 2010; Yu et al., 2013).

Neofusicoccum australe, apart from infecting blueberry plants, is a presence on *Rubus* sp*., Quercus suber, Prunus domestica, Accacia* sp*., Pistacia vera, Mangifera indica*, *Olea europaea*, and *Vitis vinifera* (Armengol et al., 2008; Barradas et al., 2013; Ismail et al., 2013; Linaldeddu et al., 2010; Lopes et al.,

2016; Martin et al., 2011; Phillips et al., 2006; Triki et al. 2015; Úrbez-Torres, et al., 2011).

Studies carried out by Tennakoon et al. (2017b), have revealed that *N. australe* was the dominant species infecting blueberries mostly due to their proximity to vineyards. In our study, *N. australe* isolates were obtained from symptomatic material, alone from branches with dieback and in association with other *Botryosphaeriaceae* species from dead branches; and from asymptomatic material, as endophyte, co-existing with *N. parvum*, and *Colletotrichum* in the same branch. Although these two *Neofusicoccum* species are known to be pathogens on blueberry plants, it was found that isolates from both species asymptomatically colonised the plant tissues, thus confirming them as endophytes or latent pathogens. Pathogenicity tests revealed that *N. australe* produced external and internal lesions on blueberries, but smaller than those caused by *N. parvum* (Figure 8).

As suggested by Mehl et al. (2017), among *Botryosphaeriaceae* species, *N. parvum* is a dominant species on several plants and trees and it is among the most aggressive members of this family (Iturritxa et al., 2011). Also in our study, among *Neofusicoccum* isolates, *N. parvum* showed to be the most common species found (73 %) followed by *N. australe* (15 %) and *N. eucalyptorum* (12 %). The dominance of *N. parvum* in this study, may be related with its abundance in some areas as well as associated with a wide range of hosts, mainly due to human activity that provokes an environmental disturbance (Mehl et al., 2017). This fungus has been associated to numerous hosts such as *Vitis vinifera, Protea cynaroides*, *Citrus* sp., *Eucalyptus globulus*, *Prunus dulcis, Malus domestica* and *Prunus persica* (Adesemoye & Eskalen, 2011; Barradas et al., 2016; Delgado-Cerrone et al., 2016; Iturritxa et al., 2011; Olmo et al., 2016; Phillips et al., 2002; Thomidis et al., 2011).

The occurrence of *N. parvum* on blueberries has also been described in China, Korea, Argentina, California and México (Boyzo-Marin et al., 2016; Choi et al., 2012; Koike et al., 2014; Wright et al., 2012; Yu et al., 2013). Symptoms include stem dieback, twig blight and extensive vascular discoloration (Wright & Harmon, 2010). In the present study, isolates identified as *N. parvum* were

isolated from symptomatic material, including dead branches, dieback and canker, in association with *Diaporthe*, *Pestalotiopsis* and *Botryosphaeria* species or alone. Two *N. parvum* isolates were also obtained from asymptomatic plant material thus highlighting their potential endophytic or latent pathogen nature, typical of *Neofusicoccum* species (Lopes et al., 2016).

In the combined ITS and *tef1-α* phylogenetic analysis a group of *N. parvum* isolates (M22 and M317) formed a sub-clade with reasonably high bootstrap support (88 %), meaning they could represent a distinct species. Analyses of the sequences from both loci showed no fixed nucleotide differences in the ITS and only two nucleotide differences in the *tef1-α*. This small number of differences may simply represent intraspecific variability within the species, as shown in previous studies (Lopes et al., 2017). However, to clarify if these isolates represent a novel species more loci need to be studied. For example, studies regarding the mating type genes may be considered, since Lopes et al. (2017) have proved the usefulness of *MAT* genes sequences as a phylogenetic tool for species delimitation in the genus *Neofusicoccum*.

Pathogenicity tests revealed that *N. parvum* was the most aggressive species, causing lesions significantly larger than the rest of the isolates tested (Figure 8), being the only species to cause plant death (1/3 of the inoculated plants died). Thus, *N. parvum* is possibly the main agent of dieback and canker of blueberries in Portugal. In concordance with our results are the ones from Espinoza et al. (2009) and Xu et al. (2015) that considered *N. parvum* as the most aggressive on blueberries (cv. *Bluecrop*) among other *Neofusicoccum* species.

Neofusicoccum eucalyptorum is a well-known canker-associated pathogen, apparently highly specialised on *Eucalyptus* spp. and other *Myrtaceae* species (Lopes et al., 2016; Pérez et al., 2010). However, recently, this species was reported for the first time on a host outside the family *Myrtaceae*, namely on *Fraxinus excelsior*, which belongs to the *Oleaceae*, thus raising questions about its putative host specialisation (Lopes et al., 2016).

This study reports, for the first time, the occurrence of *N. eucalyptorum* on *V. corymbosum* (*Ericaceae*) in Portugal and worldwide and consequently widening the host range of this fungal species. It can be argued that the presence of *N.*

eucalyptorum on blueberry is just coincidental as a result of high inoculum pressure, since blueberry plantations are located nearby forests densely populated with *Eucalyptus* spp. However, isolates of *N. eucalyptorum* were recovered from dead branches and branches with dieback without co-occurrence of other pathogens. Also, in pathogenicity tests *N. eucalyptorum*, although not being highly aggressive, was shown to cause lesions on blueberry plants being therefore regarded as pathogenic.

Since the spores of *Botryosphaeriaceae* species can be dispersed by wind or rain splash, the ability of these fungi to move to several hosts as well as to infect them, increases the risk to economically important crops (Mehl et al., 2017). These host jumps of *N. eucalyptorum* should not be disregarded and deserve further studies. As host jumps, should be considered an important mechanism for the emergence of new pathogens (Stukenbrock, 2013).

Diaporthe species represented a considerable number of the isolates studied (24 %). The genus has been associated with numerous woody and non-woody plants worldwide, frequently as an endophyte, but also causing a wide range of disease symptoms (Dissanayake et al., 2017; Dissanayake & Phillips, 2017). Among *Diaporthe* species, *Diaporthe* sp. 2 was the most common species found with an abundance of 32 %, followed by *D. eres* with 27 %.

A search of the SMML Fungus-Host Distribution Database (http://nt.ars-grin.gov/fungaldatabases/) retrieved several reports of *Diaporthe* species on *Vaccinium corymbosum* worldwide. Among them, we can highlight *Diaporthe rudis*, *D. australafricana*, *D. foeniculina*, *D. sterillis*, *D. eres*, *D. baccae*, *D. asheicola*, *D. vaccinii, D. ambigua* or *D. passiflorae*. These species are known to be blueberry pathogens causing stem canker and dieback worldwide (Elfar et al., 2013; Farr & Rossman, 2017; Lombard et al., 2014). Of these, only 3 *Diaporthe* species were found in our survey (*D. eres, D. foeniculina,* and *D. rudis*).

Studies conducted by Gomes et al. (2013) and Udayanga et al. (2014a) have shown that *D. eres*, presenting a large intraspecific diversity, is considered to be the most common species found associated with a wide range of families, including the *Rosaceae* (Farr et al., 2002; Santos et al., 2017)*, Vitaceae* (Cinelli et al., 2015), *Ericaceae* (Lombard et al., 2014)*, Apiaceae* (Bastide et al., 2017)*,* and

others, mostly in temperate regions worldwide (Gomes et al., 2013; Lombard et al., 2014; Udayanga et al., 2014a). However, it is not the most aggressive species (Dissanayake et al., 2017). Regarding blueberries, *D. eres* was previously isolated from plants in Germany, Poland, Lithuania and Netherlands as causal agent of stem cankers and internal discoloration of the vascular tissues (Lombard et al., 2014). In this study, *D. eres* isolates were recovered from dead branches and cankers associated with internal discoloration of the wood.

Diaporthe foeniculina, reported as causing stem canker on blueberries (Elfar et al., 2013), has also been found on *Malus domestica*, *Prunus dulcis*, *Pyrus pyrifolia, Hydrangea macrophylla, Acer negundo*, *Foeniculum vulgare* and *Diospyros kaki* as causing twig blight, twig canker and shoot blight (Diogo et al., 2010, Farr & Rossman 2017; Golzar et al., 2012; Santos & Phillips 2009; Santos et al., 2010). In the present study, we found some isolates of *D. foeniculina* occurring as endophytes in asymptomatic branches. Also, this species was isolated from dead branches, together with *D. rudis*.

Diaporthe rudis is a recognised fungal pathogen in Europe especially associated with *Vitis vinifera* (Lombard et al., 2014). Yet, this fungus has also been found on several plants in Netherlands such as *Aucuba japonica*, *Rosa rugosa*, *Lupinus* sp. and *Fraxinus excelsior* (Gomes et al*.,* 2013). It was also found in Austria, France, Canada, Portugal, China and New Zealand in *Laburnum anagyroides*, *Asphodelus albus*, *Epilobium angustifolium*, *V. vinifera*, *Citrus* sp. and *Castanea sativa*, respectively (Gomes et al*.,* 2013; Huang et al., 2015). Also, *D. rudis* has been found on blueberries in New Zealand and in the Netherlands (Lombard et al., 2014; Udayanga et al., 2014b). Despite most of the isolates from this study, identified as *D. rudis*, were isolated from plants with some kind of symptoms it was also recovered, as endophyte, from leaves with no apparent symptom of disease apart from a red or yellow colour.

Apart from *D. eres, D. foeniculina,* and *D. rudis* three putative novel *Diaporthe* species (*Diaporthe* sp. 1, *Diaporthe* sp. 2, and *Diaporthe* sp. 3) were also found. These are characterised in detail and descriptions are given for each of them. In the multi-loci analysis combing ITS*, tef1-α, tub, cal* and *his*, these three putative novel *Diaporthe* species formed clades separated from other known

species. *Diaporthe* sp. 1 and *Diaporthe* sp. 2 had very high bootstrap support (100 %) while the status of *Diaporthe* sp. 3 (representative isolate CAA762) was not entirely clear. In an initial identification based only on the ITS sequences CAA762 was identified as *D. pyracanthae*, but in the multilocus phylogeny it was placed between *D. pyracanthae* and *D. leucospermi*, but closer to this last one.

Analyses of the sequences of ITS*, tef1-α, tub, cal* and *his* of *D. pyracanthae, D. leucospermi* and *Diaporthe* sp. 3 showed that this last one shared polymorphisms with both of the previous species, differing only in two nucleotide positions in the *tef1-α* sequence. Although fungal hybrids are rare in nature they are known to occur (Stukenbrock, 2016). Thus, one hypothesis is that the isolate CAA762 may be a hybrid between *D. pyracanthae* and *D. leucospermi*. In alternative, two other possibilities may be considered. Thus, this group (*D. pyracanthae, D. leucospermi* and *Diaporthe* sp. 3) may represent a single species with high intraspecific variability or *Diaporthe* sp.3 is in fact a closely related but distinct species. Future studies using more isolates are needed to clarify the status of *Diaporthe* sp. 3. as well as the sequencing and analysis of the *MAT* genes and their comparison could be helpful (Santos et al., 2010)

Our pathogenicity tests showed that all *Diaporthe* species tested were capable of causing lesions on the inoculated plants and could therefore be regarded as pathogenic. No severe symptoms, apart from necrosis of the outer epidermis and discoloration of the internal vascular tissues, were observed. Also, no obvious difference in aggressiveness was observed between the different species tested although *Diaporthe* sp. 3 produced lesions significantly different from the lesions caused by the other species (Figure 9).

Although *Diaporthe vaccinii* has been reported on blueberry plants in southern Chile, United States, Netherlands, Poland, Lithuania, Latvia, Germany, Russia and China (Elfar et al., 2013; EPPO, 2017a; Farr et al., 2002a; Gabler et al., 2004; Jeger et al., 2017; Lombard et al., 2014; Vilka & Volvoka, 2015), in the present study, no isolates of *D. vaccinii* were found. As in the case of *B. corticis* this may be related to the low number of samples used along with a restricted sampling area. Its presence in the north of the country was reported by some authors, which detected twig blight on blueberries (Chicau, 2015; Madeira, 2016).

However, that lacks confirmation because such fact may be associated with an incorrect identification of the species as previously shown by Lombard et al. (2014). In their study, they found that isolates from blueberries were wrongly previously regarded as *D. vaccinii* since this was based only on morphological traits and host association. The occurrence of *D. vaccinii* in Portugal cannot be excluded, since Narouei-Khandan et al. (2017) have shown that the north of Portugal seems to be highly suitable for the establishment of this pathogen due to the ideal environmental conditions.

Although the focus of this study was the families *Botryosphaeriaceae* and *Diaporthaceae*, isolates belonging to 10 other fungal genera were also identified: *Alternaria*, *Colletotrichum, Neurospora*, *Trichoderma, Peyronellaea, Paraphaeosphaeria*, *Pestalotiopsis*, *Phlebia*, *Phlebiopsis*, and *Stemphylium.* Some of these are reported for the first time in Portugal associated with blueberry plants. Species from some of these genera, namely *Alternaria*, *Colletotrichum* and *Pestalotiopsis* are also known to be blueberry pathogens (Espinoza et al., 2008; Xu et al., 2013; Zhu & Xiao, 2015). Therefore, their role as pathogens of blueberry in Portugal deserves further investigation.

7. CONCLUSIONS

This study evidenced the existence of a diversity of fungal genera on *Vaccinium corymbosum* plants. Thirteen different genera, *Stemphylium*, *Phlebiopsis*, *Alternaria*, *Pestalotiopsis*, *Phlebia*, *Colletotrichum, Trichoderma, Peyronellaea, Paraphaeosphaeria*, *Neurospora*, *Botryosphaeria, Neofusicoccum* and *Diaporthe,* were identified. Within these several species, important pathogens of blueberry plants as well as potentially novel pathogens were identified.

To our knowledge, this is the first time that *Diaporthe eres*, *D. foeniculina*, *D. rudis*, *Neofusicoccum parvum*, *N. australe* and *Botryosphaeria dothidea*, known to be blueberry pathogens, were confirmed as occurring on *Vaccinium corymbosum* plants in Portugal. *Neofusicoccum eucalyptorum* was also found for the first time on blueberries, revealing to be pathogenic to this host. It is also important to highlight the existence of 3 putative novel *Diaporthe* species, identified through molecular methods and characterised morphologically.

Pathogenicity tests carried out in this study have shown that *N. parvum* is the most aggressive species on blueberries, and probably the major causal agent of dieback and canker, and eventually death of plants in Portugal. *Diaporthe* sp. 3 isolate CAA762, whose status as a novel species is unclear, showed to be the most aggressive among the *Diaporthe* species tested.

8. FUTURE APPROACHES

Large surveys on *Vaccinium* plantations including larger sampling areas as well as collecting a higher number of samples, should be considered in future studies to better understand the diversity and distribution of *Diaporthe* and *Botryosphaeriaceae* on blueberries in Portugal.

Pathogenicity tests should be performed using different cultivars to evaluate differences in susceptibility of cultivars to the identified fungal pathogens.

The use of genomes may be a future approach to understand the emergence of new plant pathogens (Yang et al., 2017). Once speciation of fungal plant pathogens and endophytes have been associated with host jumps, host domestication and hybridisation, analysing these pathogens genomes may be an advance to recognise cryptic species, to understand endophytic fungal communities and the rapid emergence of new plant pathogens, as well as to provide an insight into how pathogens cause disease on crops (Plissonneau et al., 2017; Stukenbrock, 2013; Yang et al., 2017).

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